Hepatocellular Carcinoma

Cover image: CDE or TAA-induced liver tumor. See page 78, chapter 4 for details.

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FOREWORD

There is little doubt that hepatocellular carcinoma is the new frontier in hepatology. It is a disease of global significance with a devastatingly poor prognosis and is characterized by modest therapeutic advances in the past 20 years. Much more needs to be achieved in coming years if the burden of this disease is to be reduced.

Liver cancer is an underappreciated cause of mortality on a global scale. Most hepatocellular carcinoma occurs in patients with already established liver disease. It is not surprising that there is marked regional variation in the burden of liver cancer given the geographical variation in the prevalence of liver disease, particularly viral hepatitis. Advances in the prevention and therapy of chronic viral hepatitis should have impact on the incidence of liver cancer - if these advances can be delivered to regions of greatest need. The emergence of non-alcoholic fatty liver disease as an important contributor to the incidence of liver cancer is concerning, considering the scale of the modern obesity epidemic. It is overrepresented as an underlying cause in Europe, Australasia, and high-income North America - thus illustrating the ongoing need for public health and policy interventions to target those at risk.

In this context, it is important that emerging knowledge in the field of hepatocellular carcinoma is consolidated, such that scientists and clinicians are provided with an up-to-date overview of important, evolving themes. This book is a very important contribution, in part because it draws further attention to the need for much more work in the field. Early chapters on the cellular origin of hepatocellular carcinoma and the tumor microenvironment reflect the unique pathogenesis of this malignancy and the complex array of cellular forces driving malignant change. In vitro mouse models of hepatocellular carcinoma allow those factors to be dissected out and studied in great detail. The diagnosis and management of liver cancer will, for the foreseeable future, be based on multidisciplinary input, using the experience of physicians, surgeons, and radiologists. It is therefore appropriate that the latter chapters of this book discuss contemporary issues in the care of affected patients and the need for individualized decision-making, based on the characteristics of the tumor and the patient, including the features of the underlying liver disease.

This book is a very important read for those involved in basic and translational research, as well as for clinicians delivering care to affected patients. The editor and the authors are to be congratulated on their work.

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PREFACE

Hepatocellular carcinoma (HCC) has become a major global health problem and is responsible for a steadily increasing number of cancer-related deaths. It is the most common form of liver cancer, with large disparities in incidence due to the geographical variation in the prevalence of risk factors. The high incidence rates of HCC in Africa and Asia are mainly attributed to the dietary exposure to aflatoxin B1 and chronic hepatitis B virus (HBV) infection. Western countries report fewer cases, with chronic hepatitis C virus (HCV) infection, alcohol abuse, metabolic syndrome, and non-alcoholic fatty liver disease as dominant causes. Although the risk factors for HCC development are well known and great advances have been made through HBV vaccinations, direct-acting antivirals for HCV treatment, and aflatoxin eradication programs, the overall incidence and mortality rates of HCC are still rising.

To tackle the burden of HCC, it is essential to understand the principle molecular and cellular processes as well as fundamental clinical challenges. This book aims to provide an overview on several important disease aspects. Chapter 1 reviews recent studies assessing the potential cellular origins of HCC. Chapter 2 describes the newly discovered regulatory roles of the tumor microenvironment on tumor growth and progression, with particular focus on extracellular matrix factors. Important starting points in the long pipeline from drug discovery to clinical translation of potential treatments are appropriate and well-designed models of disease that enable a thorough understanding of context-specific mechanisms. The authors of Chapter 3 and Chapter 4 have therefore outlined the most commonly used in vitro systems and animal models of chronic liver disease and HCC in great detail. Non-alcoholic fatty liver disease and non-alcoholic steatohepatitis have been growing in prevalence worldwide at alarming rates. Hence, Chapter 5 provides an overview of metabolic reprogramming and dysregulation of lipid metabolism as a newly recognized hallmark of HCC. The last three chapters focus on clinical aspects of HCC management and treatment. Chapter 6 details the currently accepted standards and challenges for the surgical management of HCC, while Chapter 7 provides an overview of the recent developments in the field of tyrosine kinase inhibitors, including survival benefits and adverse events. Finally, Chapter 8 discusses multidrug resistance to chemotherapy and potential approaches to overcome this remaining clinical obstacle.

Unmet clinical needs are most effectively addressed through close collaborations between basic researchers and clinicians, thus effectively capitalizing on each other's strengths and expertise. I therefore aimed to make this book of interest to both scientists and clinicians and provide useful insights and stimulation for constructive discussions. This project would not have been possible without the hard work and commitment of all authors. I sincerely thank everyone for their valuable contributions.

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Cellular Origin of Hepatocellular Carcinoma

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Abstract: Molecular features of hepatocellular carcinoma affect patient prognosis and treatment efficiency. This chapter provides an overview of the relevant studies conducted to identify the cell of origin of hepatocellular carcinoma with a special focus on the controversy of hepatocytes versus hepatic progenitors as the main tumor-initiating cell. Furthermore, we introduce the concept of cancer stem cells (CSCs) and highlight recent publications covering this topic in relation to liver cancer. More precisely, we concentrate on the origin of CSCs, discuss accepted markers and the need to define a consistent combination of them that can be utilized to clearly define this heterogeneous cell type, summarize important signaling pathways that govern the stemness, and describe state-of-the-art assays to isolate and evaluate CSCs. We focus on their contributions to oncogenesis and tumor heterogeneity, as well as their feature to resist chemo- and radiotherapy. Finally, the potential of using CSC markers for diagnostic purposes and therapeutic approaches targeting these cells is addressed.

Keywords: cancer stem cells; cell of origin; CSC markers; hepatic progenitor cells; tumor-initiating cell

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INTRODUCTION

Liver cancer, with hepatocellular carcinoma (HCC) representing approximately 90% of all cases, is the third leading cause of cancer-related deaths worldwide (1, 2). The main risk factors for developing HCC are well known and include chronic liver damage caused by inflammation and fibrosis, alcohol abuse, infection by hepatitis B or hepatitis C virus, metabolic syndrome, and ingestion of the fungal metabolite aflatoxin B1(1). Therapeutic approaches include surgical resection, transarterial chemoembolization (TACE), local radiofrequency ablation (RFA), and organ transplantation (3). However, most cases of HCC are diagnosed at advanced stages for which efficient therapies are limited (4). Unresectable HCC cases are treated with sorafenib, a multikinase inhibitor, with modest survival benefits (5). It is commonly known that molecular features of HCC affect patient prognosis and treatment efficiency. For example, human HCC harboring vascular endothelial growth factor A (VEGFA) gene amplification is more sensitive to sorafenib treatment (6) and in vivo RNAi screening has identified Mapk14 as a target to overcome therapy resistance (7). Therefore, it is essential to comprehensively elucidate the mechanisms underlying hepatocarcinogenesis. Over the past decade, there has been a considerable improvement in the understanding of the molecular pathogenesis of HCC (8, 9). The landscape of genetic alterations in HCC has been clearly characterized. High-level DNA amplifications were found in chromosome 6p21 (*VEGFA*) and 11q13 (fibroblast growth factor, *FGF19*; Cyclin D1, *CNND1*), as well as homozygous deletions in chromosome 9 (cyclin-dependent kinase inhibitor 2A, CDKN2A). Mutations in the telomerase reverse transcriptase (*TERT*) promoter are the most frequent, affecting 60% of HCC patients. The next most prevalent mutations are found in the tumor suppressor gene *TP53* and catenin beta 1(*CTNNB1*) (25–30%), followed by genes with low-frequency mutation rates (e.g., *AXIN1*; AT-rich interactive domain-containing protein, *ARID2*, *ARID1A*; tuberous sclerosis protein, *TSC1/TSC2*; ribosomal protein S6 kinase alpha 3, *RPS6KA3*; Kelch-like ECH-associated protein, *KEAP1*; *MLL2*). *TP53*-mutated human HCCs revealed increased Aurora A kinase (*AURKA*) expression, hypersensitivity to treatment with conformation-changing AURKA inhibitors, and a positive correlation between *AURKA* and the proto-oncogene *MYC* expression (10). These findings help to define some of the core deregulated pathways in HCC (8, 11). The role of chronic tissue damage, inflammation, and metabolism, as well as signaling pathways controlling the immune response during hepatocarcinogenesis, has been extensively studied (12–20).

Yet, there is still a need to gain a much deeper insight into the mechanisms responsible for liver cancer initiation; that is, the cellular origin; and progression; that is, propagation and maintenance; to facilitate the detection of more reliable tumor markers for diagnostic and prognostic applications, and the development of new targeted therapy approaches for liver cancer.

In this chapter, we will review relevant studies conducted to identify the cell of origin of HCC with a special focus on the controversy of hepatocytes versus hepatic progenitors as the main tumor-initiating cell (TIC). Furthermore, we will introduce the concept of cancer stem cells (CSCs) and highlight recent publications covering this topic in relation to liver cancer. More precisely, we will concentrate on the origin of CSCs, discuss accepted markers and the need to define a

consistent combination of them that can be utilized to clearly define this heterogeneous cell type, summarize important signaling pathways that govern the stemness, and describe state-of-the-art assays to isolate and evaluate CSCs. We will focus on their contributions to oncogenesis and tumor heterogeneity, as well as on their feature to resist chemo- and radiotherapy. Finally, the potential of using CSC markers for diagnostic purposes and therapeutic approaches targeting these cells will be addressed.

CELL OF ORIGIN OF HCC

HCC is highly heterogeneous in cellular morphology, genetic landscape, and response to therapeutic interventions (21, 22). Two major molecular clusters (proliferation and non-proliferation) with distinguishing enrichment in prognostic signatures, pathway activation, and tumor phenotype have been identified (8). Interestingly, one subtype of the more aggressive proliferation class was specifically enriched in markers of progenitor cells (23, 24). These observations have led to several hypotheses about the cell(s) of origin of liver cancer with hepatocytes and hepatic progenitor cells (HPCs) as the main cellular elements whose malignant transformation could initiate hepatocarcinogenesis. Many studies carried out in the last years have attempted to shed light on the controversy of the origin of the TIC. In general, mouse primary HPCs, lineage-committed hepatoblasts, and differentiated adult hepatocytes were shown to be targetable by oncogenic transformation and to enable tumorigenesis via activation of diverse cell-specific pathways (25) (Figure1). However, the nature of target cells affected susceptibility to transformation, tumor histopathology, and global gene expression profiles. Tumors of HCC-like pattern predominantly derived from mature adult hepatocytes underlined that tumorigenic cells keep at least part of the differentiation program typically seen in the original cell, while HPC tumors adopt a more primitive mesenchymal-like state (25). Of importance, distinct genetic changes are needed for the oncogenic transformation of different hepatic lineage cells. In addition, the type of genetic alteration predisposing towards carcinogenesis further contributes to the phenotypic and molecular diversity of HCC. In non-transformed HPCs as well as hepatocytes, loss of the tumor suppressor p53 resulted in chromosomal imbalances and increased clonogenic capacity, and formation of tumors with bilinear differentiation after transplantation into immunocompromized mice (26). In the following sections, we will discuss the different evidences supporting HPCs or hepatocytes as the cellular origin of HCC (Table 1).

Hepatic progenitor cells as tumor-initiating cells of HCC

Over the past years, several genetic and chemically induced HCC preclinical mouse models have been established (27–30). In fact, some of them suggest a progenitor cell origin of liver tumors (Figure 1, Table 1). HPCs isolated from mouse embryos were able to generate liver carcinomas resembling human HCC after isolation and ex vivo genetic manipulation followed by transplantation into the livers of recipient mice (31). Progenitor cells in mouse liver were shown to give rise to cancer due to interleukin-6 (IL-6)-driven transformation accompanied

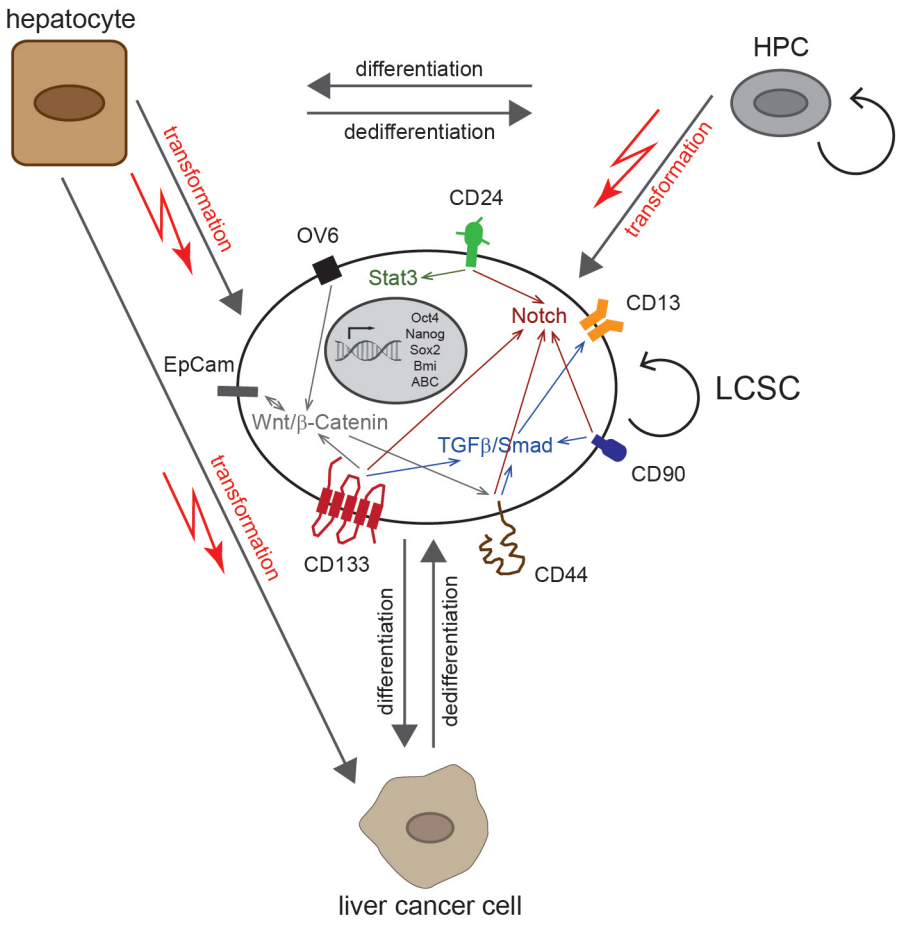

Figure 1 LCSCs—origin and characterization. Hepatocytes, hepatic progenitor cells (HPC) and differentiated liver cancer cells are potential cellular origins of liver cancer stem cells (LCSC) via transformation or dedifferentiation. Different markers were shown to be specific for LCSCs. Signaling pathways associated with these markers are depicted.

by inactivated transforming growth factor beta (TGFβ) signaling (32). In contrast, constant TGFβ stimulation in cirrhotic liver was shown to promote the neoplastic transformation of HPCs to hepatic TICs that facilitate hepatocarcinogenesis through an miR216a/phosphatase and tensin homolog (PTEN)/Akt-dependent pathway (33). Both studies support a role of HPCs as the cell of origin of HCC but point to a contradictory role for TGFβ during their malignant transformation, potentially due to the interaction with other signaling pathways.

Mice with attenuated Hippo signaling activity within the liver expanded progenitor cells and subsequently showed liver tumor formation (34). These findings are also relevant to human liver cancer, where the majority of human HCCs show elevated levels of nuclear yes-associated protein (YAP), which is indicative of attenuated Hippo signaling in these tumors (35, 36). Similarly, deletion of the tumor

TABLE 1 The different hypotheses about the cell of origin of HCC

Proposed cell of origin	Model	Pathways	Reference
HPCs	Orthotopic transplantation into C57BL/6 mice	cIAP1, YAP	(31)
	elf$^{+/-}$ mice	IL-6, TGFβ	(32)
	Xenotransplantation into NOD-SCID mice	TGFβ, Akt	(33)
	mst1/2 and sav1 conditional mutant mice	Hippo	(34)
	Xenotransplantation into nu/nu mice	Nf2/Merlin	(37)
	AFP-NICD mice	Notch	(38)
	Ctnnb1 conditional mutant mice	Wnt	(40)
	P240 PR-SET7$^{\Delta HepA}$ mice	STAT3	(43)
	DEN/2-AAF/PH treatment of F-344 rats	AP-1/JUN	(44)
	2-AAF/PH treatment of F-344/N Slc rats	retinoid receptors	(45)
	Epcam^{CreERT2} transgenic mice DDC Xenotransplantation into NOD-SCID mice	Wnt	(46)
Hepatocytes	Tsc1/Sqstm1$^{\Delta hep}$ and Sqstm1$^{\Delta hep}$/MUP mice	p62/NRF2/mTORC1/ c-Myc	(48)
	Stat3$^{f/f}$ mice, Il6$^{-/-}$ AAV injection db/db mice, Mdr2$^{-/-}$ mice	IL-6/STAT3	(49)
	R26Tom Hnf1b^{CreER} transgenic mice DEN, Mdr2$^{-/-}$ mice	N/A	(50)
	Opn^{iCreERT2} Rosa26R^{YFP} transgenic mice Rosa26$^{loxP\text{-}mTom\text{-}stop\text{-}loxP\text{–}mGFP}$, Rosa26$^{loxP\text{-}stop\text{-}loxP\text{–}ZsGreen1}$ Cre reporter mice AAV injection DEN, DEN/CCl_4, DEN/CDE, DEN/DDC, Mdr2$^{-/-}$ mice	N/A	(51)
	Alfp^{Cre} p53$^{fl/fl}$ hydrodynamic tail-vein injection	YAP, Wnt	(52)
	Foxl1^{Cre} Rosa26R^{YFP} transgenic mice Rosa26$^{loxP\text{-}stop\text{-}loxP\text{-}YFP}$ Cre reporter mice AAV injection DEN/CCl_4, DEN/TCPOBOP	YAP	(53)
	SOX9IRES^{CreERT2} Rosa26R^{YFP}, serum albumin (SA)^{CreERT2} Rosa26R^{YFP} transgenic mice hURI-tetOFF^{hep}mice	galectin-3, α-ketoglutarate	(54)

suppressor gene neurofibromatosis type 2 (Nf2) in livers of developing or adult mice resulted in liver cancer formation that was preceded by a progressive expansion of progenitor cells while differentiated hepatocytes were not affected (37).

Notch signaling, activated in one-third of human HCCs, was shown to promote liver carcinogenesis in a genetically engineered mouse model (38). All Notch-induced tumors showed various degrees of nuclear staining for the Notch target gene SOX9, a marker of HPCs (39); and SOX9 overexpression was frequently observed in human HCCs. Therefore, during hepatocarcinogenesis, Notch may either control the expansion of a pre-existing progenitor-like cell population or drive progenitor-like properties to differentiated cells (Figure 1).

Furthermore, somatic β-catenin stabilization in a unique population of progenitor cells in fetal liver resulted in the frequent development of HCCs with spontaneous lung metastases (40). Interestingly, this is in striking contrast to the absence of tumors when β-catenin is stabilized in adult hepatocytes indicating that activation of the Wnt pathway alone is insufficient for HCC initiation. Indeed, additional introduction of genetic alterations such as oncogenic Ha-rat sarcoma (Ras) or Akt mutation does result in cancer formation (41, 42).

Mice with proliferation-deficient hepatocytes spontaneously developed hepatic tumors composed of cells with CSC characteristics, including the capacity for self-renewal, differentiation, and tumorigenesis, due to prolonged necrotic regenerative cycles combined with oncogenic signal transducer and activator of transcription (STAT) 3 activation (43). The highly proliferating cancerous cells in this model can only be derived from HPCs that are still capable of proliferation and differentiation.

The investigations of hepatocarcinogenesis in different rat models additionally point towards HPCs as a potential cell of origin of HCC. Comprehensive characterization of the neoplastic development, by exploring the expression of the biliary and HPC marker cytokeratin (CK) 19 during the evolution of early preneoplastic lesions to fully developed HCC, suggested the potential progenitor derivation of the majority of the developed tumors (44). Additionally, global gene expression analysis revealed that CK19 may serve as a prognostic marker of early persistent hepatic preneoplastic lesions. Moreover, a CK19-associated gene signature discovered through comparative functional genomics robustly stratified HCC patients according to clinical outcome, highlighting the strength of this rat model to reproduce stem cell/progenitor cell-derived human HCC (44). Subsequently, a subpopulation of precancerous cells in another rat liver carcinogenesis model was identified, which were enriched in $CD133^{+}CD44^{+}$ cells that formed part of the HPC fraction (45).

Finally, a recent lineage-tracing analysis showed that HPCs activated in chronically damaged liver and thought to originate from proliferating ductal cells were specifically labeled in epithelial cell adhesion molecule (EpCAM) CreERT2 mice and gave rise to HCCs through the accumulation of induced genetic alterations, supporting the existence of progenitor-derived hepatocarcinogenesis (46).

Hepatocytes as the cellular origin of HCC

More recent studies highlight adult hepatocytes as the other main source of HCCs (Figure 1, Table 1). These cells have the potential to directly transform into cancer cells following sequential genomic damage and dedifferentiate into precursor cells

expressing markers of progenitor cells (47). It was shown that hepatocyte-specific p62 expression promotes c-MYC induction, mechanistic target of rapamycin (mTORC) 1 activation, and HCC initiation (48). Another investigation demonstrated that mice overexpressing FGF19 in hepatocytes develop HCC (49). Moreover, activation of STAT3 signaling through induced IL-6 production in the hepatic microenvironment was shown to be essential for FGF19-induced tumorigenesis. Both studies demonstrate that genetic targeting of hepatocytes promotes development of liver cancer in mice.

In contrast to the lineage-tracing analysis employing EpCAMCreERT2 mice described above, studies using various other fate-tracing systems have shown that in hepatotoxin-induced as well as in carcinogen-free models, HCC does not originate from progenitor cells, thereby clearly demonstrating that tumors arose from hepatocytes in the liver. Tracking of progenitor cells via their expression of the biliary marker hepatocyte nuclear factor (HNF) 1β provided the first clear evidence that tumors in classical genotoxic or genetic mouse HCC models do not originate from HPCs (50), at least in these experimental animal models. Consecutively, complementary fate-tracing approaches were employed to label the progenitor compartment and hepatocytes in murine hepatocarcinogenesis in order to not only rule out that HPCs represent the cell of origin of HCC but also prove that indeed hepatocytes bear the TICs. Tracking HPCs through osteopontin (Opn)-CreERT2 and genetically labeling of hepatocytes via infection with adeno-associated viral serotype 8 (AAV8)-thyroxine binding globulin (Tbg)-Cre suggested that hepatocytes constitute the main cellular source of HCC in mice and that a progenitor signature may not reflect progenitor origin, but dedifferentiation of hepatocyte-derived tumor cells (51). Indeed, loss of p53 facilitated YAP-induced tumorigenesis. Mature hepatocytes dedifferentiated into nestin-positive progenitor-like cells, followed by differentiation into HCCs in response to mutations targeting Wnt (52). Utilizing a complementary strategy to label the HPC compartment, Forkhead box L1 (Foxl1)+ cells, which express the progenitor markers EpCAM, SOX9, and CD133, were shown to not contribute to HCC tumorigenesis (53). Here, tumors arose exclusively from hepatocytes. Using human data as well as mouse models of HCC, HPCs were shown to be activated and expanded by transformed hepatocytes through galectin-3, maintaining HPC stemness, and α-ketoglutarate, preserving an HPC undifferentiated state (54). In the human unconventional prefoldin RPB5 interactor (hURI)-tetOFF^{hep} mouse model, both hepatocytes and HPCs contributed to tumor heterogeneity. However, HCC predominantly originated from hepatocytes, whereas benign lesions developed from HPCs (54). Of note, HPCs are mainly activated and start to proliferate in damaged livers where hepatocyte proliferation is compromised (55). Most experimental conditions often do not actively suppress the ability of hepatocytes to proliferate and may therefore not always reflect the diverse human settings, which may well favor HPC proliferation due to local hepatocyte inhibition.

LIVER CANCER STEM CELLS

The observation that tumors exhibit significant cellular heterogeneity with respect to their tumorigenic potential led to the CSC concept (56). This concept proposes that the growth of tumors is fuelled by limited numbers of dedicated stem cells

that are capable of unlimited self-renewal and production of heterogeneous progeny (57). CSCs are considered to be highly tumorigenic, metastatic, chemotherapy- and radiation-resistant, and responsible for tumor relapse (58). Indeed, the participation of liver cancer stem cells (LCSCs) in hepatocarcinogenesis was reported. Initial studies were based on the identification of a side population (SP) in HCC cell lines and tumors after staining with the DNA-binding dye Hoechst 33342 that was enriched in cells displaying CSC properties (59, 60). Interestingly, when DEN-induced collagenase-resistant aggregates were isolated and characterized, cells were detected that can give rise to HCC only after transplantation into an appropriate host liver undergoing chronic injury (61). These HCC progenitor cells (HcPCs) acquired autocrine IL-6 signaling that stimulated their in vivo growth and malignant progression. Ectopic lymphoid structures (ELS), associated with chronic nuclear factor "kappa-light-chain-enhancer" of activated B-cells (NF-κB) activation, were shown to function as cytokine-rich microniches for these tumor progenitor cells (62).

Subsequent investigations focused on the attempt to identify and use reliable membrane marker(s) for LCSCs. In the following sections, we will discuss accepted markers and the need to define a consistent combination of them that can be utilized to clearly define this heterogeneous cell type, summarize important signaling pathways that govern their stemness, and describe state-of-the-art assays to isolate and evaluate CSCs.

Markers to identify LCSCs

CD133, also referred to as prominin-1, is a well-established cell surface marker of hematopoietic stem cells, neuronal stem cells, and HPCs (63). In HCC, its presence seems to be of clinical significance, since patients with high CD133 expression exhibit poor overall survival and higher recurrence rates compared with patients with low CD133 expression (64). A meta-analysis of all the data available in the literature about the correlation between CD133 expression and various clinicopathological parameters revealed that the abundance of CD133 expression correlated with enhanced alpha-fetoprotein levels, a poor histological grade and survival, but did not show significant relation with hepatitis, cirrhosis, and stage of the tumor (65).

Moreover, CD133 was identified as a LCSC marker. Initial studies were based on the identification and characterization of $CD133^+$ cells in hepatocarcinoma cell lines. In $CD133^+$ cells, when compared to their $CD133^-$ counterpart, a greater colony-forming capacity in vitro and higher proliferative activity as well as enhanced ability to form tumors in vivo, both in orthotopic and subcutaneous cancer models, was seen (66–68). $CD133^+$ cells preferentially expressed genes associated with stemness, such as Bmi1, SOX2, Oct4, Notch, Nanog, Nestin, and membrane transporters ATP-binding cassette (ABC) G2 and ABCB1 (68). Subsequent studies focused on the characterization of $CD133^+$ cells in primary human hepatocarcinomas. CD133 expression in HCC was associated with an advanced tumor stage, a larger tumor size, and a poor prognosis (69). A rare CD133 population in HCC specimens, with expression ranging from 1.3 to 13.6% of the total tumor cell population, was identified (69). When isolated, these cells were able to form tumor spheroids composed of undifferentiated tumor cells and

had a larger capacity to grow tumors of identical morphology compared to the parental ones in immunodeficient mice (69). Interestingly, overexpression of miR-130b, an miR targeting p53-induced nuclear protein 1 (TP53/NP1), was detected in $CD133^+$ cells. To further characterize these cells on a molecular level, $CD133^+$ and $CD133^-$ cells were isolated from both tumor cell lines and primary tumor samples and characterized by genome-wide expression analysis. Self-renewal, tumorigenesis, and angiogenesis were shown to be promoted by $CD133^+$ liver TICs through neurotensin-induced activation of the IL-8 and chemokine (C-X-C motif) ligand 1 (CXCL1) signaling cascade (70).

CD44, a major adhesion molecule of the extracellular matrix and the receptor for hyaluronic acid, is implicated in a wide range of biological processes. CD44 potentiates AKT activation, thereby ceasing the p53 genomic surveillance response. DNA-damaged hepatocytes thus escape p53-induced death and senescence and respond to proliferative signals, promoting the accumulation of mutations and subsequently transformation to HCC progenitors (71). In HCC, the expression of CD44s (CD44 standard variant) was related to TGFβ-mediated regulation of the mesenchymal phenotype, and a negative patient prognosis was associated with overexpressed levels of CD44s (72). CD44s was recently shown to play an important role in maintaining CSCs and regulating oxidative stress of an HCC cell line in a Notch3-dependent manner. In addition, CD44 expression in HCC tissues was significantly correlated with Notch3 expression, further strengthening the idea that CD44 regulates CSC properties via Notch3 (73). In an effort to investigate interactions between the tumor microenvironment and CSCs, IL-6 produced by tumor-associated macrophages (TAMs) was shown to promote expansion and tumorigenesis of $CD44^+$ cells. Concomitantly, levels of IL-6 in human HCC samples positively correlated with tumor stage and markers of CSCs (74).

In a separate study, CD44 was preferentially expressed in the $CD133^+$ population, and double-positive cells possessed the abilities of extensive proliferation, self-renewal, and differentiation. Furthermore, double-positive cells expressed more stem cell-associated markers, such as Bmi1, rendering them highly tumorigenic and chemoresistant (75). Moreover, $CD133^+CD44^{high}$ cells played a key role in hematogenous metastasis of liver cancers, with CD133 being responsible for tumor growth and CD44 being important for invasion (76). In human patients, $CD44^+$ and $CD133^+$ correlated with increased risk of poorly differentiated HCC and elevated alpha-fetoprotein levels. CD44 and CD133, alone or in combination with microvascular invasion, are independent predictors of poor prognosis in patients undergoing transplantation for HCC (77).

Expression levels of *CD24*, a mucin-like cell surface glycoprotein, are related to liver cancer progression and prognosis (78). Additionally, it was recently identified as a potential marker of LCSCs. $CD24^+$ HCC cells were found to be critical for the maintenance, self-renewal, differentiation, and metastasis of tumors through STAT3-mediated Nanog upregulation, and to significantly impact patients' clinical outcome. CD24 expression overlaps with that of CD133 and EpCAM (79). CD24 expression on hepatocarcinoma cells was shown to be induced by Twist2 and to be required for the stimulation of HCC stem cell self-renewal (80). Most recently, an investigation of the regulation of CSCs by the tumor microenvironment demonstrated that HGF and IL-6 secreted by cancer-associated fibroblasts promoted self-renewal, chemotherapy resistance, metastasis, and tumorigenicity

of $CD24^+$ cells. More precisely, regulation of stemness properties was dependent on STAT3 signaling (81). The abundance of the stem cell markers CD24 and CD133 in tumors of HCC patients correlated with increased inducible nitric oxide synthase (iNOS) expression, promoting Notch1 signaling and subsequent development of stemness traits, as well as accelerated HCC initiation in the mouse xenograft tumor model (82).

EpCAM, a homophilic, $Ca2^+$-independent cell–cell adhesion molecule, is expressed on a subset of normal epithelia and overexpressed on malignant cells derived from a variety of tumors. This overexpression is even more pronounced on TICs (83). HCCs can be subdivided into two different subgroups, with $EpCAM^+$ tumors displaying features typically observed at the level of HPCs and $EpCAM^-$ hepatocarcinomas exhibiting features more typical of mature hepatocytes (24). EpCAM expression was induced by Wnt-β-catenin (84). Moreover, zinc finger protein X-linked was shown to activate and maintain $EpCAM^+$ liver CSCs by promoting nuclear translocation and transactivation of β-catenin (85). $EpCAM^+$ cells isolated from $EpCAM^+AFP^+$ HCCs displayed properties of CSCs and were able to initiate tumorigenesis when inoculated into immunodeficient mice (86, 87). The highest tumor-initiating activity in hepatocarcinoma cell lines was found in $CD133^+EpCAM^+$ cells, compared to $CD133^+EpCAM^-$ and $CD133^-EpCAM^+$ populations (88).

CD90, a glycosylphosphatidylinositol-anchored glycoprotein, also known as Thy-1, was revealed to be a reliable marker for CSCs. The number of $CD90^+$ cells isolated from different HCC cell lines positively correlated with tumorigenicity and metastatic potential. $CD45^-CD90^+$ cells, in contrast to $CD90^-$ or $CD45^-CD90^-$ cells, isolated from tumor tissues and blood samples of liver cancer patients had the capacity to generate tumor nodules in immunodeficient mice (89, 90). Interestingly, CD44 was shown to regulate the survival and the tumorigenic activity of $CD90^+$ liver cancer cells. $CD90^+CD44^+$ cells showed a more aggressive phenotype than the $CD90^+CD44^-$ counterpart (90). In primary HCC, EpCAM and CD90 expressions were mutually exclusive. Gene-expression analysis of sorted cells suggested that $EpCAM^+$ cells exhibited features of epithelial cells, whereas $CD90^+$ cells resembled mesenchymal cells (91). A poorly differentiated morphology and high serum alpha-fetoprotein was associated with the presence of $EpCAM^+$ cells, whereas a high incidence of distant organ metastasis correlated with CD90 positivity (91). Most interestingly, a potential interaction of $EpCAM^+$ and $CD90^+$ CSCs was demonstrated. The motility of $EpCAM^+$ cells was enhanced by $CD90^+$ cells when cocultured in vitro through the activation of TGFβ signaling (91). CyclinD1 overexpression and subsequent Smad signaling increased the development of the $CD90^+EpCAM^+$ cell population, concomitantly increasing stemness and chemoresistance (92). Studying gene expression differences between $CD90^+$ CSCs from tumor tissue and $CD90^+$ cells from non-tumorous counterparts confirmed the upregulation of genes in $CD90^+$ CSCs associated with the biological processes of liver inflammation, chemoresistance, and lipid metabolism (93).

Enrichment of *CD13*, a membranous glycoprotein, was correlated with early recurrences and poor prognosis in patients with HCC (94). It was identified as a marker for semi-quiescent CSCs in human liver cancer cell lines and clinical samples (95, 96). The association of $CD13^+$ CSCs with a hypoxic marker in clinical HCC samples points to a critical role of these cells in carcinogenesis and resistance to therapy in liver cancers (97). In liver cancer cells, increased CD13

expression was associated with TGFβ-induced epithelial to mesenchymal transition (EMT), concurrently preventing further increases of both reactive oxygen species levels and the induction of apoptosis, thereby promoting the survival of $CD13^+$ cells (98).

OV6, a monoclonal antibody isolated from carcinogen-treated rat liver, was shown to serve as a hepatic progenitor marker (99). Interestingly, the expression of this molecule defined a subpopulation of less differentiated progenitor-like cells in both HCC cell lines and primary HCC tissues (100). These cells exhibited endogenously active Wnt/β-catenin signaling, enhanced tumorigenicity in vivo, and a substantial resistance to standard chemotherapy (100). CSC-like HPC lines overexpressing OV6 as well as CD133, EpCAM, and the pluripotency factor Oct4 can be established from human non-tumorous, tumor-surrounding tissue (101). Moreover, OV6-positive TICs were more invasive and metastatic both in vitro and in vivo and expressed high levels of C-X-C chemokine receptor type 4 (CXCR4), indicating a role for SDF-1/CXCR4 signaling in sustaining stem cell properties (102). Patients with elevated numbers of $OV6^+$ tumor cells were associated with aggressive clinicopathologic features and poor prognosis (102).

Marker combinations to clearly define LCSCs

As discussed above, several LCSC markers have been reported and used to isolate and characterize LCSCs (Figure 1). However, the reliability of each of these markers in identifying true LCSCs is still controversial (103) calling for a comprehensive evaluation of the effectiveness of stem cell markers. In an effort to evaluate the efficiency of some markers to characterize and isolate LCSCs, a range of the most commonly used ones (CD44, CD90, and CD133) were tested in both human HCC samples and HCC cell lines. Surprisingly, CSC markers were present in both tumors and adjacent non-cancerous liver. However, the number as well as the staining intensity of positive cells varied with no consistent expression patterns (104). Furthermore, LCSCs isolated from the same cell line via different markers or from different cell lines via the same markers exhibited a unique genetic program of gene expression reflecting the strong heterogeneity of the origin of liver cancer and possibly the varied etiology of HCC (104). On the contrary, a more recent study demonstrated that increased expression of a combination of markers (CD90, CD24, CD13, and CD133) in HCC not only correlated with advanced disease stage but also with larger tumor size and worse overall survival (105). The markers CD90, CD44, CD133, CD13, and CD24 were present diversely in all HCC samples. In contrast to the previous study, their expression in non-tumor liver tissues was almost absent (105). $CD90^+CD24^+CD13^+CD133^+$ HCC cells possessed progressively increasing self-renewal and tumor-initiating ability in vitro and in vivo (105).

Combining more than one marker has been shown to increase the isolation efficiency (75, 103). LCSCs most probably represent a large group of diverse subtypes, each expressing their own different combination of markers. To develop future therapies targeting CSCs, and to predict prognosis and efficacy of these therapies, it is therefore crucial to comprehensively study and define these distinct groups of CSCs in relation to their expression profiles and clinicopathologic features of HCC.

Signaling pathways governing stemness of LCSCs

Several signaling cascades in LCSCs are important to regulate their capacity of unlimited self-renewal and production of heterogeneous progeny, their tumorigenic and metastatic potentials, as well as their resistance to chemotherapy and radiation (Figure 1). The essential pathways are Wnt/β-catenin, Notch, and TGFβ, among others (106).

Wnt/β-catenin activation is one of the pathways being aberrantly active in HCC (107). Proliferation, rapid generation of tumor spheres, and high invasiveness of SP cells isolated from liver cancer samples depended on Wnt/β-catenin signaling (108). In this group of liver cancer cells, elevated expression of β-catenin leads to an increased expression of Wnt/β-catenin target genes, including AXIN2, DKK1, and CCND1 (108). Importantly, activation of Wnt/β-catenin signaling has been reported in $CD133^+$(68), $EpCAM^+$(86), and $OV6^+$(100) CSCs. The Wnt pathway is activated following nuclear translocation of the β-catenin component, thereby inducing the transcription of prominent targets, such as CD44 (109), EpCAM (84), and c-Myc (110).

Notch signaling misregulation in liver cancer has been described as both oncogenic and tumor-suppressive, depending on the cellular context (111). This pathway was activated in $CD90^+$ cells isolated from HCC cell lines and was associated with self-renewal, invasion, migration and expression of stem cell-related genes (112). Notch signaling stimulated G1-S transition in the cell cycle phase and inhibited apoptosis, thus facilitating CSC features (112). $CD90^+CD24^+CD13^+$ $CD133^+$ HCC cells utilize upregulation of Notch and Wnt/β-catenin to initiate tumor growth and self-renewal (105).

Activation of the sonic hedgehog (Shh) pathway occurs in the $CD133^+$ subpopulation of Hepa 1–6 cells that harbor stem cell features (113). In general, Hh–Notch interactions were shown to regulate cell-fate decisions in an HPC-like cholangiocyte cell line (114).

TGFβ serves as a central regulator of signal transduction during inflammation and HCC (115). Recently, TGFβ signaling has also been linked to the malignant transformation of LCSCs. The percentage of SP cells, as well as their survival rate and chemotherapeutic resistance, was shown to increase following TGFβ treatment of a hepatoma cell line. Gene analysis revealed that epidermal growth factor receptor (EGFR) was upregulated and that this was dependent on Smad (116). On the contrary, TGFβ treatment resulted in decreased cell survival and concomitantly a reduced number of SP cells in HCC cell lines through induction of accumulation of cells at G0/G1 and upregulation of p-c-Jun N-terminal kinases (JNK), p-c-Jun, and p-Smad2 expression(117). These recent results indicated that TGFβ has anticancer effects mediated by inhibition of CSC survival. Differences in the analyzed cell lines and assays most probably account for the diverse outcomes. Nevertheless, both studies emphasize the diverse and controversial functions of TGFβ signaling in LCSCs. CD133 expression was upregulated by TGFβ1 stimulation through epigenetic regulation of promoter methylation. Furthermore, increased tumorigenicity of TGFβ1-induced $CD133^+$ cells compared to $CD133^-$ cells was shown (118). A change in the expression pattern of stem cell genes, enhancement of their stemness potential, and migratory and invasive capacity was observed in HCC cells, mediated by TGFβ-induced EMT (119). Similarly, HIF1α-induced EMT, by activation of the Notch1

pathway through direct interaction with Notch intracellular domain, promoted the CSC characteristics of HCC cells (120). When investigating the tumor microenvironment, TAMs were found to secrete TGFβ1 that promoted CSC-like properties through EMT induction (121).

Both HCC cell lines and HCC patient samples were shown to exhibit expression of at least one key driver of embryonic development such as Oct4, Nanog, SOX2, and STAT3 accompanied by the expression of genes of the Wnt/β-catenin and TGFβ families (122). Highly enriched CSC populations isolated from different liver cancer cell lines maintained a common gene expression signature characteristic of cellular stemness and harbor an activation of NF-κB as well as IL-6 and Wnt/β-catenin signaling pathways. Each individual cell line typically exhibited an activation of unique oncogenic pathways such as EGFR, MYC, and SRC, which are known to be associated with HCC (123).

Isolation of LCSCs

Currently, identification and isolation of LCSCs is achieved through several approaches, including (i) detection of SP by the Hoechst 33342 exclusion assay (59), (ii) separation using surface markers (124), and (iii) in vitro tumor sphere formation (125, 126). SP cells can be detected and isolated by flow cytometry through their ability to efflux Hoechst 33342 dye through an adenosine triphosphate (ATP)-binding cassette (ABC) membrane transporter. Overexpression of ABC proteins was associated with CSCs, conferring drug resistance to them (127). SP cells purified from HCC cells were shown to harbor CSC-like properties (59). However, some restrictions are associated with this isolation approach, since the SP compartment contains both stem and non-stem cells, and, on the other hand, other stem cells of ill-defined identity are not found in the SP fraction (128). Interestingly, epigenetic modulation increased the frequency of cells with CSC properties in the SP fraction isolated from human cancer cells, facilitating functional isolation of cells, which possess self-renewal and tumor-initiating capacity (123).

LCSCs are commonly isolated from cell cultures or whole liver by fluorescent (or magnetic) activated cell sorting using surface markers reported to be specific for CSCs of HCC. As already discussed above, the heterogeneity and complex nature of CSC biology hamper the reliable use of single—or even combinations of—markers to draw reproducible conclusions.

Sphere cultures have been used as a method for the enrichment of stem cells relying on their property of anchorage-independent growth. The tumor sphere-forming cells derived from human hepatoma cell lines were capable of proliferation and self-renewal, and possess higher tumorigenicity and a general resistance to chemotherapeutics (126). Using this approach may favor the selection of a specific subpopulation of CSCs during cultivation.

The different ways to isolate LCSCs all have their limitations, and therefore, caution has to be taken when comparing results obtained with dissimilar approaches. In the future, improved knowledge of the diversity of LCSCs will allow to define and selectively isolate these cells. CSC-specific properties, that is, unlimited self-renewal, ability to develop a malignant tumor, and resistance to chemotherapeutic agents, can be evaluated by some assays in vitro and in vivo.

Characterization of LCSCs

The clonogenic or colony formation assay represents an in vitro cell assay based on cell survival and the ability of an individual cell to grow into a colony, thereby testing for the ability to divide an unlimited number of times (129). This assay can provide information about cell survival and resistance after treatment with different agents. However, this assay does have limitations such as the loss of the three-dimensional environment of a cell within a given tissue. Therefore, the effect of cell–cell or matrix–cell communication on cell proliferation cannot be measured. Moreover, this assay cannot be used in case the substance concentration decreases cell growth but does not affect cell cycle progression and/or DNA synthesis (130).

The ability to form spheres is used to enrich CSCs and can additionally also be applied as an in vitro method for assessing the self-renewal and multipotency capacity of a given cell population. Three-dimensional spheroids can be formed by CSCs containing a heterogeneous population of progenitor cells, which can differentiate into multiple cell types under these low-adherence and non-differentiating conditions. The ability of cells to form tumor spheres upon multiple passages demonstrates the self-renewal capacity of CSCs, and this potential correlates with the number of spheres formed (106). Hypoxia and the low pH in the sphere's core and the characteristics of the inner cells that may be inaccessible to metabolites and drugs in comparison to exterior cells are believed to mimic the characteristics of solid tumors in vivo. Moreover, this assay has been used to evaluate the migration and invasive ability of CSCs. Even though self-renewal of CSCs can be usefully assessed by this assay, several limitations have to be acknowledged (131). The size of generated spheres and the number of cells that are necessary to form spheres strongly depend on the cell type and methodology used. This makes the comparison of results from different cell types challenging (132).

In an in vivo tumorigenicity assay, the tumor cell population of interest is transplanted into animal models, followed by an evaluation of their tumor-propagating capacity (133). Nevertheless, this assay has some limitations. This relates to the use of immunodeficient animals and the fact that the context of tumor development is clearly different from recipient animals harboring a normal immune system. Additionally, it is important to consider that upon xenotransplantation, the architecture and stroma of the tumor differ compared with its native niche. Finally, if the cells used for transplantation are isolated based on a selection of markers, the effects of the total population can be lost. Some of these constraints can be circumvented by using syngeneic models, by injecting the cells orthotopically, or by analyzing different subpopulations simultaneously with the total population, although this is not always possible. The transplantation assay is the current "gold standard" for identifying CSCs because it can assess both self-renewal and multipotency. On the other hand, lineage tracing is the current gold standard for defining the cell of origin of transformation in mouse models. However, it is also being applied to elucidate the proliferative potential and fate of stem cells (125). Different cell-specific promoters allow distinct cell subpopulations to be labeled, facilitating tracking of a single cell-derived clone in animals. Lineage tracing utilizes (in some cases inducible) Cre transgenic mouse lines, harboring cell type-specific gene promoters to drive Cre expression, and common reporter lines, either fluorogenically or colorigenically flanked by a loxP-STOP-loxP sequence. Cre expression via excising loxP-STOP-loxP cassettes activates the

reporter in cells that possess the respective promoter activity. As with other assays, there are limitations associated with lineage tracing as well. Labeling efficiencies are highly variable depending on the Cre- or reporter-driving promoters. Systems frequently become "leaky," having minor but detectable Cre activity in the absence of the inducer, resulting in spontaneous background recombination. One of the main limitations is the fact that CSCs or HPCs are a particular heterogeneous population that may switch phenotype and marker expression (134) in a context-dependent manner. Therefore, lineage tracing of these cells focuses on a certain subpopulation only. Nevertheless, when carefully considering all potential pitfalls, this assay presents a valuable tool to obtain a better understanding of the cellular origins of cancer and CSCs (125). Although in vitro assays are convenient and faster, until now, the best assay to reliably and robustly assess tumorigenicity has been in vivo evaluation.

THERAPEUTIC RESISTANCE OF LCSCS

The effectiveness of standard therapies against HCC, such as chemotherapy, the multikinase inhibitor sorafenib, and radiotherapy, is impaired by LCSC-mediated resistance (135) (Table 2). Cellular quiescence, DNA repair capacity, and ABC-transporter expression are characteristics of CSCs mediating chemo- or radiotherapy resistance and regrowth of the tumor after treatment (128). The increased expression of stem cell surface proteins in liver cancer SP cells induced the rapid formation of tumor spheres and enhanced transcription of drug efflux genes (ABCG2, MDR1, and ABCB5). These cells were resistant to numerous DNA targeting drugs (108). $CD133^+$ HCC cells contributed to chemoresistance through preferential activation of Akt/PKB and Bcl-2 cell survival response, thereby supporting the opinion that CSCs are the source of tumor recurrence after chemotherapy (136). Additionally, these cells were more resistant to radiation-induced apoptosis than $CD133^-$ cells and exhibited greater proliferation and tumor-initiating capacity in vivo post-radiation (137). Downmodulation of this membrane antigen in isolated cells induced both a decrease in their stemness properties and an enhancement in their chemo- and radiosensitivity, at least to some extent, indicating that resistance of $CD133^+$ liver CSCs is related to CD133 expression (138). An enrichment of $CD90^+$ and $CD133^+$ cells was observed in tumor spheres obtained from the culture of HCC cell lines under serum-free conditions favoring stem cell growth. These spheres showed a high overexpression of ABCG2 and Oct4 and resistance to chemotherapy drugs (127). Expression of CD13 was shown to reduce the extent of DNA damage induced by the production of reactive oxygen species following genotoxic stress, thereby protecting cells from apoptosis, and thus rendering cells radio- and chemoresistant (95, 96).

In a study that focused on exploring whether CSC markers have a predictive role with regard to the sorafenib response in HCC patients, overexpression of CD133 and CD90 in HCC was associated with a worse response to the multikinase inhibitor and therefore a shorter progression-free survival time (139). Sorafenib-resistant HCC tumor cells show a high expression of CD24. The requirement for resistance to sorafenib of this functional marker relied on AKT/mTOR-mediated autophagy regulation (78).

TABLE 2 Therapeutic resistance in LCSCs

LCSC marker	Resistance	Mechanisms	Reference
Side population cells	5-FU, gemcitabine, oxaliplatin, paclitaxel, cisplatin, etoposide, oxaliplatin	ABCG2, MDR1, ABCB5	(108)
	5-FU	TGFβ/Smad/EGFR	(116)
	Sorafenib	AKT, ERK	(142)
CD133	Doxorubicin, fluorouracil	Akt/PKB	(136)
	Radiation	MAPK/PI3K	(137)
	Cisplatin, doxorubicin, radiation	Bcl-2/Bax	(138)
	Sorafenib	ABCG2	(168)
CD133, EpCAM	Doxorubicin	N/A	(88)
CD133, CD90	Doxorubicin	Oct4, ABCG2	(127)
	Sorafenib	N/A	(139)
CD133, CD44	Sorafenib	ABCC1–3	(140)
CD24	Sorafenib	AKT/mTOR	(78)
	Cisplatin	STAT3/Nanog	(79)
	Sorafenib	STAT3	(81)
CD90	Doxorubicin	PI3K/Akt1	(169)
CD90, EpCAM	Cisplatin, doxorubicin	cyclin D1/Smad	(92)
EpCAM	Sorafenib	TSC2/AKT	(141)
CD13	5-FU, doxorubicin, radiation	N/A	(95–97)
OV6	Cisplatin	Wnt/β-catenin	(100)
Sphere-forming cells	Cisplatin, 5-FU, gemcitabine, mitomycin, sorafenib	N/A	(126)
Chemo-resistant cells	5-FU, cisplatin, doxorubicin	Oct4/AKT/ABCG2	(170)

Enriched proportions of $CD44^+$ and $CD44^+CD133^+$ HCC cells in sorafenib-resistant cells, as well as upregulation of stemness genes Nanog, SOX2, and Oct4 in EpCAM-positive HCC cells and enhancement of tumorigenicity after treatment with sorafenib (140, 141), further suggest that sorafenib can foster cancer stemness in liver cancer. A subpopulation of CSCs derived from HCC cell lines, referred to as label-retaining cancer cells that are distinguished by pluripotency gene expression profile, were shown to possess a relative resistance to sorafenib. Treatment of these CSCs led to reduced apoptosis and improved viability and was accompanied by gene expression profiles, which mark stem cell differentiation (142). All results emphasize the role of sorafenib treatment in CSC maintenance and CSC-mediated resistance against sorafenib.

CLINICAL IMPLICATIONS OF LCSCS

The important role of LCSCs in the initiation, maintenance, relapse, metastasis, and drug resistance of HCC has been identified. Therefore, development of novel liver cancer diagnosis and treatment strategies will be impacted by the identification of signaling pathways as well as stem cell markers activated in LCSCs (58). Targeting LCSCs is expected to be a promising approach for the treatment of liver cancer (143).

HCC patients with stemness-associated gene expression traits generally have a poor prognosis (23, 24, 123, 144, 145). However, predictive values of single LCSC markers still remain controversial (146). Rather, a combination of several markers may provide greater specificity and reliability in predicting HCC prognosis (24, 147). CSCs can be isolated from peripheral blood mononuclear cells as circulating tumor cells due to their highly invasive and metastatic capacity and thus may provide diagnostic or prognostic information (89, 148).

In recent years, targeting LCSCs has become a novel strategy to improve the outcome of HCC treatment. Targeted therapies based on tumor cell-specific cell surface markers have been proposed to specifically eradicate LCSCs (149). Anti-CD133 antibody-drug conjugates inhibited $CD133^+$ HCC growth in vitro and in vivo (150). Similarly, CD44 blockade prevented the formation of local and metastatic tumor nodules by the $CD90^+$ cells (90), and EpCAM blockage via RNA interference significantly inhibited cellular invasion, spheroid formation, and tumorigenicity of an HCC cell line (86). Additionally, it was shown that the combination of a CD13 inhibitor and the genotoxic chemotherapeutic fluorouracil (5-FU) reduced tumor volume compared with either agent alone. 5-FU inhibited $CD90^+$ proliferating CSCs, some of which produced $CD13^+$ semiquiescent CSCs, while CD13 inhibition suppressed the self-renewing and tumor-initiating ability of dormant CSCs (95), suggesting that combining a marker-targeted treatment with a chemo- or radiation therapy may improve the treatment of liver cancer.

Some promising targets against LCSCs for the treatment of HCC can be found among the several signaling pathways that are essential for the development and maintenance of LCSCs (143). Constitutive expression of Wnt/β-catenin was detected in LCSCs, and downregulation of it suppressed the cell phenotype (108). Employing different inhibitors of this pathway clearly impaired the viability of LCSCs as well as decreased the tumorigenicity in vitro and in vivo (151–153). Moreover, some phytochemicals have also been demonstrated to restrain the self-renewal and proliferationof LCSCs by suppressing Wnt/β-catenin signaling (154, 155). Lupeol, another phytochemical, inhibited chemoresistance, self-renewal ability, and tumorigenicity of $CD133^+$ CSCs, concomitantly sensitizing these cells to chemotherapeutic drugs via the PTEN-Akt-ABCG2 signaling pathway (156). Usage of a small molecule inhibitor targeting TGF-β/Smad signaling followed by conventional therapy induced CSC differentiation, resulting in significant chemosensitization in vitro and in vivo (92).

Another interesting therapeutic approach is the induction of CSC differentiation into non-CSCs to lose their self-renewal property (149). Oncostatin M (OSM), an IL-6-related cytokine, is known to enhance differentiation of hepatoblasts into hepatocytes by inducing the activation of the STAT3 pathway (157). OSM effectively induced the differentiation of $EpCAM^+$ LCSCs. Moreover,

combining oncostatin M treatment and 5-FU-based chemotherapy efficiently targeted both CSCs and non-CSCs and ultimately eliminated HCC (158). HNF4α is a key transcription factor for hepatocyte differentiation. Differentiation of hepatoma cells, especially CSCs, into hepatocytes could be induced by forced re-expression of this protein, which was associated with a decrease in stemness gene expression and the relative abundance of $CD133^+$ and $CD90^+$ cells (159). Arsenic trioxide also induced cell differentiation, consequently sensitizing LCSCs to conventional chemotherapy in HCC (160). All-trans retinoic acid effectively induced differentiation of TICs, which potentiated the cytotoxic effects of cisplatin (161). High-dose exogenous BMP4 promoted $CD133^+$ LCSC differentiation and inhibited the self-renewal, chemotherapeutic resistance, and tumorigenic capacity of these cells (162). In addition, inducing differentiation of already premalignant hepatic cells via blocking of mCXCL1 was proposed as a novel therapeutic strategy in HCC (163).

One of the recent approaches to target CSCs directly involves immunotherapies. Chimeric antigen receptor T cell (CAR-T) targeted against glypican-3 (an attractive liver cancer-specific target as it is highly expressed in HCC but displays limited expression in normal tissues) was shown to suppress HCC growth (164). CSC antigen-targeted CAR-T cells are therefore promising tools for the direct eradication of these cells.

Although numerous strategies for targeting LCSCs have been investigated, treatments for the eradication of CSCs still require further development until they are suitable to enter the clinics. Potential adverse effects on normal stem cells should be carefully evaluated because CSCs share similar features with normal stem cells. Therefore, the future challenge is to identify specific CSC markers and develop a specific treatment for LCSCs.

CONCLUSION

To improve diagnosis, prognosis, and treatment of HCC, it is of uttermost importance to get a much broader and deeper knowledge about the cancer-initiating cell as well as the cancer-propagating cell. For human cancer, the target cell population of malignant transformation is controversially discussed, but increasing evidence suggests that different cells of origin (Figure 1) as well as diverse genetic mutations account for cancer heterogeneity (58). More recent state-of-the art lineage tracing studies employing different models of experimental hepatocarcinogenesis highlight the role of hepatocytes as the cellular origin of HCC (Table 1). Not only these studies proved that tumors originated almost exclusively from hepatocytes but also ruled out a direct involvement of HPCs in initiating carcinogenesis. Nevertheless, most of the investigations were performed in animal models which have some limitations. Considering the fact that during chronic liver injury a variety of cells can respond to the need for cell replacement and liver regeneration (165–167), it is highly likely that the cell of origin of HCC is equally context-specific. Therefore, it is crucial to further strengthen the examination of human HCC, to identify the cells that give rise to liver tumors and elucidate the different classes of tumors based on their molecular features.

Liver cancers with stemness traits are generally associated with a poor prognosis for patients, indicating that CSC markers have both diagnostic and prognostic potential. LCSCs are typically resistant to chemo- or radiotherapy as well as sorafenib treatment (Table 2) and have been shown to play critical roles in tumor progression, maintenance, and recurrence. Targeting surface markers or signaling pathways (Figure 1) in, or inducing differentiation of, these cells has already been demonstrated to interfere with tumorigenicity in preclinical studies. Although these data are promising, there are still some obstacles to overcome before similar strategies can enter the clinics. Specificity is one major concern since CSCs share identical features with normal stem cells that can be only resolved by unequivocally characterizing LCSCs. So far, the lack of a uniform definition of the CSC (sub) populations complicates the reliable comparison of results obtained using different approaches to isolate and characterize these cells. Furthermore, LCSCs are likely to be distinct and different for each individual tumor, according to genetic traits and activated signaling pathways. To define therapeutic targets specifically aimed at LCSCs, it is essential to face this challenge and consistently work on the elucidation of traits that confer CSC properties.

To conclude, the cellular mechanisms responsible for liver cancer initiation and progression need to be clearly defined to facilitate the detection of reliable tumor markers for diagnostic and prognostic applications and the development of new targeted therapy approaches for liver cancer.

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2

The Role of the Tumor Microenvironment in the Development and Progression of Hepatocellular Carcinoma

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Abstract: There is growing evidence that supports the role of the tumor microenvironment in the development and progression of hepatocellular carcinoma. The tumor microenvironment is composed of cellular components, bioactive substances, and extracellular matrix comprising of proteins such as collagens, proteoglycans,

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and the linear glycosaminoglycan hyaluronan. Hepatocellular carcinoma generally arises from fibrotic or cirrhotic liver, characterized by alterationin extracellular matrix components. In addition, non-tumoral cells such as mesenchymal stem/stromal cells (MSCs) are typically recruited to the injured or hypoxic area within the tumor. Besides the secretion of immunoregulatory proteins, growth factors, and cytokines, MSCs and hepatic stellate cells can also synthesize hyaluronan, amongst other components, which affects several tumor-associated processes. The tumor microenvironment also contains different types of immune cells. A key component in the genesis of hepatocellular carcinoma is the macrophages, as tumor-associated macrophages (TAM). This chapter provides an overview of the interaction of MSCs-hyaluronan-TAMs and tumor cells, and how this interaction potentially contributes to the development and progression of hepatocellular carcinoma.

Keywords: hepatocellular carcinoma; hyaluronic acid; macrophages; mesenchymal stem cells; tumor microenvironment

INTRODUCTION

The biology of a tumor can only be understood by studying different cell types within the tumor microenvironment (TME) (1). The interaction between tumor cells and the associated stroma plays a crucial role in the initiation and progression of a tumor (2). The heterogeneity of tumors is based not only on the genomic profile but also on their microenvironment composition (2). The microenvironment actively regulates tumor initiation, its progression, metastasis, and therapy response (3). The extracellular matrix (ECM), as part of the TME, is essential for asymmetric cell division and maintenance of tissue polarity; it may block or facilitate cell migration, determine the direction of cell–cell communication, and bind to growth factors to prevent their free diffusion (4). Changes in ECM support the development of hepatocellular carcinoma (HCC), and the complexity of TME and therapeutic failures may be explained, in part, by alterations of components of the ECM. The development of HCC is associated with prolonged inflammation caused by chronic virus infection, alcoholic exposure, or metabolic diseases. The inflammatory microenvironment facilitates the transformation of normal liver cells such as hepatocytes, stem, immune, and stellate cells by providing a suitable environment for the development and progression of a tumor (5, 6). HCC is a primary liver tumor that derives, in most cases, from hepatocytes and corresponds to approximately 90% of all liver cancers (7, 8). Since cholangiocarcinoma, hepatoblastoma, and angiosarcoma are less common than HCC, they are not discussed in this chapter.

THE TUMOR MICROENVIRONMENT

The TME is composed of non-cellular and cellular components (4). The ECM is the non-cellular component. The cellular component, apart from tumor cells, consists of a variety of cells including tumor-associated fibroblasts (TAFs),

angiogenic endothelial cells, bone marrow-derived cells, adipocytes, and cells of the immune system (Figure 1) (9). In HCC, hepatic stellate cells (HSCs) are also part of this cellular microenvironment (10). The bidirectional interaction between the tumor and its microenvironment greatly affects tumor initiation, progression, and drug resistance, and a better understanding of this interaction may enable the identification of novel targets for tumor therapy (11, 12).

Non-cellular compartment

During embryonic development and organ homeostasis, the composition of ECM is tightly regulated. However, in diseases such as cancer, it is usually deregulated

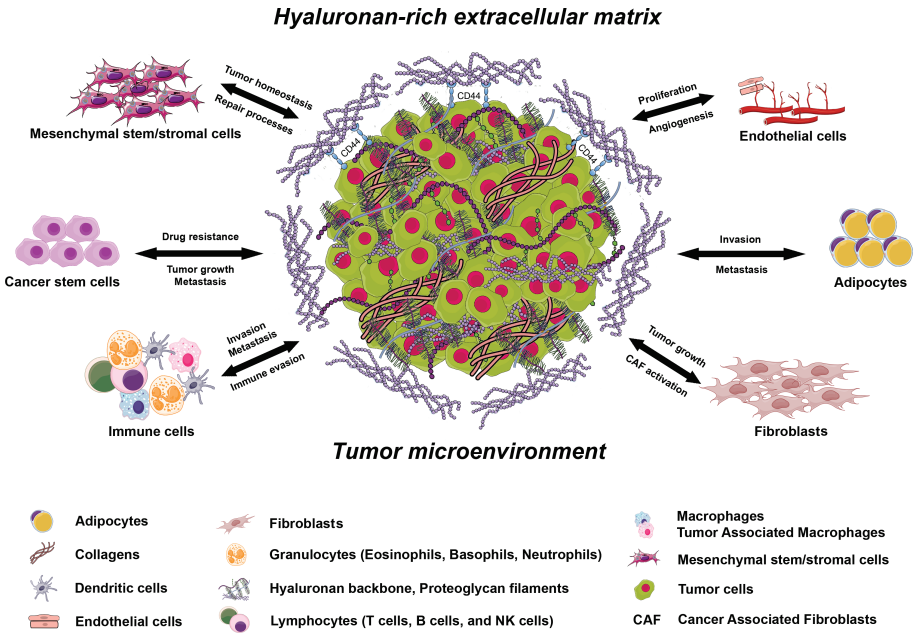

Figure 1 Schematic representation of the role of a HA-rich microenvironment in cancer progression. The TME is composed of non-tumor cells, such as fibroblasts, endothelial cells, MSCs, adipocytes, and infiltrating immune cells, and of non-cellular compartments, including secreted soluble factors and solid-state structural ECM. HA is an abundant component of the ECM that recruits and activates stromal cells to stimulate cell proliferation, migration, differentiation, angiogenesis, immune effects, and therapy resistance. HA induces intracellular signals through several receptors, mainly CD44, whose expression is associated with the characteristics of CSCs. Accumulation of HA in the tumor stroma drives the differentiation and activation of CAFs. CSCs are described as tumor initiators and are associated with tumor proliferation, drug resistance, and metastasis, whereas some cells such as MSCs can be integrated into the TME after recruitment and interact with tumor cells to promote tissue homeostasis and repair processes. The TME contains several types of immune cells including macrophages, neutrophils, dendritic cells, granulocytes, and lymphocytes. TAMs usually have a pro-tumoral action since they can promote tumor neovascularization and have an immunosuppressive action. CAF, cancer-associated fibroblasts; CSC, cancer stem cells; ECM, extracellular matrix; HA, hyaluronan; MSC, mesenchymal stem/stromal cells; TAM, tumor-associated macrophages; TME, tumor microenvironment.

and disorganized. Abnormal ECM alters the behavior of stromal cells and, as a consequence, supports and leads the generation of the TME (4). One of the components of ECM that is altered in tumors is the glycosaminoglycan hyaluronic acid (HA). HA is a linear molecule composed of disaccharide units of N-acetyl glucosamine and glucuronic acid; it is synthesized by hyaluronan synthases and degraded by hyaluronidases and glycosylphosphatidylinositol (GPI)-anchored hyaluronidase PH-20 (13, 14). Activities of these enzymes are shown to greatly influence tumor growth and metastasis (15). HA is overexpressed in both cirrhotic and liver tumor tissues, promoting tumor progression (16, 17). Several pieces of evidence indicate that HA inhibition by 4-methylumbelliferone (4-MU), a specific HA synthesis inhibitor, delays HCC growth (18, 19). Besides, the use of recombinant hyaluronidase as an adjuvant therapy in different types of cancer shows the complex relationship between hyaluronan synthases and hyaluronidases in maintaining HA expression (20). HA is an abundant component of the ECM that mediates cell proliferation, migration, and differentiation during inflammation and tumor development. Most malignant tumor tissues contain elevated levels of HA compared to their normal counterparts (21). Remarkably, HA levels rise in the serum of patients with liver injury, and it is proposed as a biomarker for high-score fibrosis and cirrhosis (16). HA is a ubiquitous molecule with high concentrations found in the synovial fluid, vitreous humor, skin, and umbilical cord. At homeostasis, HA is mostly present in a high molecular weight form, ranging from $0.5x10^6$ to 10^7 Da, and to a lesser extent in a low molecular weight form, ranging from 10^4 to $0.5x10^6$ Da. The low molecular weight form is mostly present in pathological conditions such as inflammation and cancer (22, 23). HA acts by inducing intracellular signals through several receptors: toll-like receptor 4, lymphatic vessel endothelial hyaluronan receptor 1, and receptor for hyaluronan-mediated motility (24, 25). The main receptor, CD44, is also considered a marker of cancer stem cells (CSC). It is encoded by the *CD44* gene, which is a large and highly conserved gene (20 exons, out of which 10 can undergo alternative splicing). It has been demonstrated that the interaction between HA and CD44 promotes tumor progression in different solid tumors, including HCC (14, 26).

Proteoglycans (PGs) are composed of at least one linear negatively charged polysaccharide chain, such as heparan sulfate, chondroitin sulfate, keratan/dermatan sulfate or heparin, that is covalently attached to a core protein (27). In healthy tissues, PGs are essential for structural scaffolding in the ECM, interactions with cytokines and growth factors and their receptors, and inducing cell signaling (28). During carcinogenesis, the expression of PG is markedly altered to promote cancer cell growth, survival, adhesion, migration, and angiogenesis (28).

Cellular components

Several types of cells belonging to the TME have been described as key regulators of different aspects of the tumor process. CSCs are described as tumor initiators and are associated with tumor growth, drug resistance, and metastasis (29). HSCs are key cells in responding to the inflammatory state in the liver and are the principal cells that promote ECM remodeling (30), whereas MSCs can be attracted into the TME and, after recruitment, can interact with tumor cells to promote tumor modifications (12, 31). CSCs have a constant interaction with their specific

microenvironments called niches. CSC niches are formed by different cellular components and regulated by secreted factors such as cytokines and growth factors (12). CSCs exhibit the capacity for self-renewal, pluripotency, tumorigenicity, and resistance to therapy. Many cancer therapies eliminate most of the tumor cells but ultimately fail because they do not eliminate CSCs fully, which survive to regenerate new tumors. CSCs possess several intrinsic mechanisms of resistance to current chemotherapeutic drugs (1, 32, 33). They have a high-level expression of ATP-binding cassette (ABC) transporters, which are correlated with multidrug resistance. ABC transporters reduce the cellular accumulation of various types of therapeutic agents, and therefore, CSCs become more resistant to even higher doses of anti-tumor agents (34).

MSCs represent a heterogeneous population of multipotent progenitors first described in bone marrow but present in almost all vascularized organs. Due to their high plasticity, they show various functions according to the requirements of that particular tissue. These include, among others, homing to sites of tissue damage, the initiation of repair processes, and the regulation of tissue homeostasis. Tumor growth usually induces tissue remodeling, creating an inflammatory environment. Consequently, MSCs can be recruited to these tumor sites and activated to have repair and immunomodulation functions. Several factors such as interleukin (IL)-8, monocyte chemoattractant protein-1, growth-regulated oncogene, and autocrine motility factor, produced by the HCC, are known to attract and recruit MSCs (35, 36). It is known that MSCs can secrete several growth factors, cytokines, chemokines, and ECM components (37). Once within the tumor, direct and indirect interactions between MSCs, the ECM and cancer cells increase plasticity within the tumor tissue and its microenvironment.

The TME also contains several types of immune cells such as macrophages, neutrophils, dendritic cells, T cells, regulatory T cells (Tregs), natural killer (NK) cells, and eosinophils (37). Studies have shown that changes in the number and function of these immune cells contribute to the development, tolerance, and progression of HCC (38–43). Macrophages are the major component of the immune infiltrate that is present in tumors (44, 45). Several studies indicate that tumor-associated macrophages (TAMs) usually have a pro-tumoral action, since they can stimulate angiogenesis, increase tumor cell invasion and motility, and have an immunosuppressive action (44, 45). In the case of HCC, TAMs, as infiltrated monocytes and resident Kupffer cells, are characterized as the most important immune cell type that promotes tumor invasion and metastasis (37).

THE TUMOR MICROENVIRONMENT IN HCC DEVELOPMENT

Hepatocarcinogenesis is a multifactorial process. Most HCC cases are associated with alcohol abuse, nonalcoholic steatohepatitis (NASH), and chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) inducing an inflammatory process followed by regeneration. Persistent hepatic injury and concurrent regeneration could produce an environment that eventually leads to the formation of hypoxia and inflammation, which are crucial features of HCC microenvironment (5, 6, 46). HCC has a heterogeneous population of CSC, which are considered to be tumor-initiating cells. It has been reported that 28–50% of HCC cells express

progenitor cell markers (47). Many potential origins of hepatic CSCs have been described. They may result from genetic and epigenetic modifications of hepatocytes, hepatic oval cells/liver progenitor cells (LPCs), or circulating bone marrow cells. These transformed cells, in combination with deregulated microenvironment, result in a distinct lineage of CSCs that have stem-like features (Figure 2). Some cell surface markers for CSCs include CD44, CD133, CD90, CD105, CD45, CD13, and epithelial cell adhesion molecule (EpCAM) (5). CSCs have a very complex signaling network that includes crosstalk with different non-tumoral cells. During tumor development, multiple immunosuppressive molecules are released from cancer cells, which subsequently contribute to the establishment of an immunosuppressive TME (5, 48). LPCs are small cells (7–10 μm in diameter) with basophilic character. They have small ovoid nucleus and a high nuclear-cytoplasmic ratio. LPCs are heterogeneous, hardly detectable in healthy liver, but

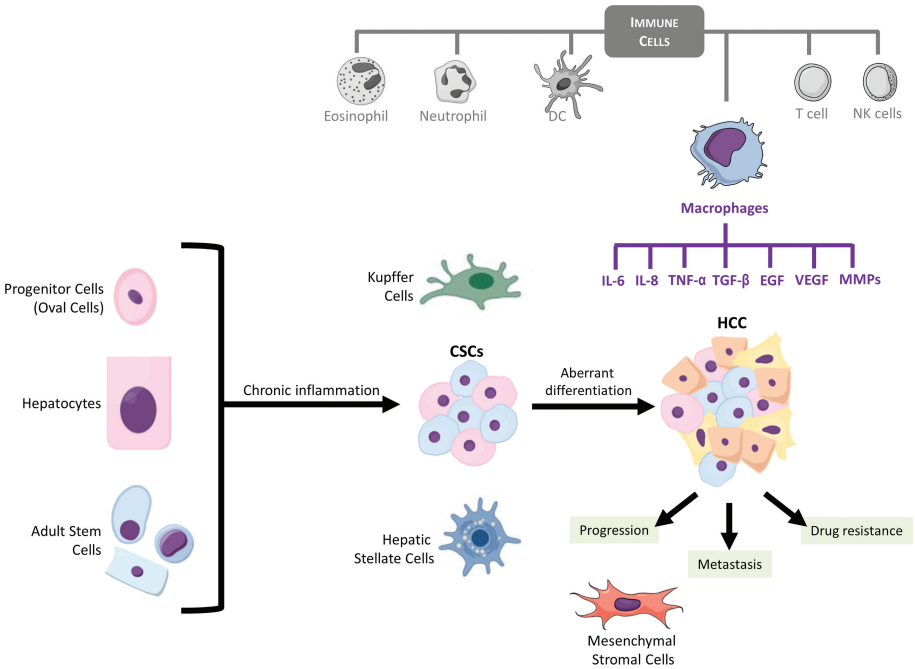

Figure 2 Stem and immune cells associated with tumor development. HCC is composed of a heterogeneous population of CSCs, which might derive from hepatocytes, progenitor cells (oval cells), or other adult stem cells, like bone marrow cells. CSCs have a very complex signaling network that includes crosstalk with different non-tumor cells, such as immune cells. The tumor microenvironment contains several types of non-tumor cells: macrophages, Kupffer cells, stellate cells, dendritic cells, T cells, Tregs, and NK cells. Changes in the number and function of these cells contribute to the development of immune tolerance and progression of HCC. Tumor-associated macrophages are characterized as the most important immune cell type that promotes tumor invasion and metastasis. Similar to cancer cells, macrophages such as Kupffer cells secrete several types of cytokines and factors crucial for HCC progression, metastasis, and drug resistance. CSC, cancer stem cells; EGF, epidermal growth factor; HCC, hepatocellular carcinoma; IL-6, interleukin 6; IL-8, interleukin 8; MMPs, matrix metalloproteinases; MSC, mesenchymal stem/stromal cells; NK, natural killer; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; Tregs, regulatory T cells; VEGF, vascular endothelial growth factor.

are activated in chronic liver injury. The origin of these cells is still debatable. The inhibition of LPCs correlates with reduced tumor development, and their activation and proliferation are linked to HCC development. In addition, they have been implicated in hepatocyte regeneration (49, 50).

The role of MSCs in tumor initiation is still controversial, particularly in HCC. In vitro evidence indicates that during MSC differentiation into hepatocytes, aberrant activation of Wnt/β-catenin is associated with a tumoral phenotype, involving increased proliferation, elevated proliferating cell nuclear antigen expression, cell cycle alteration, and spheroids formation (51). Another report suggests that MSCs may initiate HCC. The HCC cell line SK Hep-1 has been shown to display MSCs-like features and the capacity to differentiate into osteogenic and adipogenic lineages (52). Although these in vitro data indicate the potential role of MSCs in hepatocarcinogenesis, in vivo evidence to clarify this potential process is lacking.

Chronic inflammation is a risk factor for the development of tumors (53). HCC frequently arises in chronically inflamed liver. Sustained inflammation is characterized by a continuous activation of immune cells that release free radicals that can damage the DNA and cause a neoplastic transformation. The TAMs derived from Kupffer cells or circulating monocytes are recruited into the tumor tissues by chemokines and other factors secreted by tumor cells and the inflammatory cells present in the TME (37). TAM-derived cytokines and growth factors play a key role in the initiation of HCC. One of the most important TAM-derived cytokines is IL-6, which triggers pathways that promote proliferation and survival of hepatocytes, stimulating the initiation and development of HCC. It has been reported that IL-6$^{-/-}$ mice had lower incidence of HCC tumors and longer survival than wild-type mice (54).

The changes in ECM and its components allow the tumoral transformation of hepatocytes. It has been observed that patients with liver fibrosis and advanced cirrhosis present high levels of HA in serum (16). In an experimental model that mimics liver injury or fibrosis (18), HA was detected in injured/fibrotic liver but not in normal tissues. HA is synthesized by the synovial lining cells, HSCs, and MSCs during wound healing of the liver (16). HA is also associated with the stem cell niche. The ECM of this microenvironment is composed of HA among other components such as laminin, collagen, sulfated chondroitin-sulfate, and heparin-sulfate proteoglycans that maintain stemness (55). Liver injury induces the expression of HA; during the chronic process, HA elevation is continuous, allowing the interaction with the potential cancer stem cell marker CD44, which actively promotes tumor initiation (56). Lee et al. showed that HA-based multilayer films mimicked the stem cell niche and selected and enriched for liver CSCs (57). Besides, HA could be involved in HCC initiation, given its association with IL-6 expression. Particularly in cirrhotic liver, IL-6 is highly produced by Kupffer cells, and together with other inflammatory mediators, IL-6 has the ability to induce HSC trans-differentiation to myofibroblasts (58, 59). Moreover, IL-6 is essential for the expansion of mutated hepatocytes (60). It has been reported that IL-6 binds selectively to HA, suggesting that this retention and concentration near the site of secretion favor its paracrine and autocrine activities, contributing to tumor development. In addition, the inhibition of HA by 4-MU decreases IL-6 production in TME significantly, reducing tumor growth (18, 61). Recently, in a model of HBV-transgenic mice, the inhibition of HA by 4-MU was accompanied by a

reduction of CSC markers CD44, CD133, CD90, and EpCAM during hepatocarcinogenesis (62).

Other key players in cancer pathogenesis are PGs. Tumoral tissues have differential PG expression patterns, which are closely associated with their differentiation and biological behavior. Furthermore, during liver carcinogenesis, HSCs become activated; they proliferate and synthesize excess ECM proteins in most types of chronic liver diseases (63). Decorin is a member of the small leucine-rich proteoglycan (SLRP) gene family, containing a single chondroitin sulfate (CS) or dermatan sulfate chain, and is expressed by fibroblast and myofibroblasts (64). Syndecan molecules (syndecan-1, syndecan-2, syndecan-3, syndecan-4) are a major family of cell-surface heparin sulfate (HS) PGs. They mainly bear HS chains, although some members can be additionally substituted with CS chains (65, 66). In healthy liver, decorin levels are generally low. However, an increased decorin expression was observed in the connective tissue septa during fibrogenesis and in chronic liver injury (67). In this process, decorin colocalizes with high amounts of transforming growth factor beta 1 (TGF-β1), which is a key stimulator of fibrogenesis (68). In normal human liver, syndecan-1 is expressed in sinusoidal endothelial cells (69). As cirrhosis progresses, syndecan-1 expression is increased, and its localization extended to the entire hepatocyte membrane surface and expressed on the surface of biliary epithelial cells (70). Elevated syndecan-1 expression appears to be more closely associated with liver cirrhosis, rather than malignant transformation (65).

THE TUMOR MICROENVIRONMENT IN HCC PROGRESSION AND METASTASIS

HCC is known to harbor different populations of cancer cells with stem cell properties, which can be identified by different cell surface markers, such as EpCAM, CD44, CD90, and CD133. Some studies have shown that $EpCAM^+$ and $CD90^+$ cells are two independent subpopulations. $EpCAM^+$ cells have hepatic epithelial stem cell features and are associated with a high tumorigenic capacity, while $CD90^+$ cells have mesenchymal-vascular endothelial cell features and metastatic propensity. On the other hand, it has been shown in HCC cell lines that express CD133 participate in cell survival through the regulation of glucose uptake and autophagy. These studies suggest that $CD133^+$ CSCs could use autophagy to escape the selective pressure of nutrient deficiency and the hypoxic environment in HCC (71–73). CSCs originating from LPCs were found to have differential expression of a number of microRNAs (miRNAs). These miRNAs were mostly implicated in angiogenesis, post-transcriptional protein modification, and small molecule metabolism. Differential expression of miRNAs demonstrates crucial roles of LPCs during the progression of HCC (71, 73). Several signaling pathways, including Wnt/β-catenin, BMI-1, TGF-β, Notch, and Hedgehog, are known to be stem cell regulators and to accelerate tumorigenesis. These, as well as some additional factors such as EpCAM, Lin28, or miR-181, interact with CSCs and enhance the progression of HCC (6, 71, 72). On the other hand, CSCs also benefit from other processes such as angiogenesis. In fact,

HCC is one of the most vascularized solid tumors with particular vascular anomalies (48, 72).

Once a tumor is established, MSCs can be recruited from a distant place of the same organ or peripheral tissues (e.g., bone marrow) into the TME. Studying the function of the recruited MSCs on the tumor development has been of great interest during the past decade. Studies that co-injected mice with exogenous MSCs (isolated from bone marrow, adipose tissue, or umbilical cord from healthy donors) and tumor cells produced equivocal results. While some reports indicated that MSCs promoted tumor development, others demonstrated that MSCs were able to inhibit tumor growth (74). The discrepancies of results could be related to several factors including the tumor type, the heterogeneity in MSC (source, donor age, culture conditions), and the timing at which MSCs are introduced into the TME. These discrepancies remain true for HCC as well. The first reports indicated that MSCs inhibited HCC growth in vitro and in vivo (75, 76). However, other results demonstrated either a pro-tumorigenic effect (77, 78) or a null effect of MSCs on HCC growth (35, 36, 79–82). The inhibition of tumor growth was associated with Wnt, NF-κB, and PI3-K/Akt signaling pathways (75, 83), whereas enhancement of microvessel density was observed in the case of tumor progression (77, 78). Not only MSCs but also their secretome affect HCC development. Conditioned medium from human fetal MSCs expressed insulin growth factor binding proteins that could bind to insulin-like growth factors (IGFs). This leads to reduced IGF-1R and PI3K/Akt activation and induces cell cycle arrest (84). Extracellular vesicles derived from human bone marrow-derived MSCs have also been demonstrated to inhibit HCC growth in vitro and in vivo (85, 86).

The role of MSCs in tumor metastasis has also been studied. Li et al. demonstrated in a subcutaneous model of HCC that MSC-treated mice exhibited larger tumors but a decreased number of lung metastases. This effect seemed to be related to TGF-β1 downregulation (87). Moreover, repeated inoculation of MSCs in a mouse model of high metastatic HCC resulted in an inhibitory effect on HCC growth at 3 weeks after MSC engraftment and downregulation of metastasis-related factors (88). It was also described that MSCs exposed to an inflammatory microenvironment promoted HCC metastasis through TGF-β-induced epithelial-mesenchymal transition (EMT) in tumor cells (89). Efforts have been made to isolate and characterize MSCs from HCC tumors. Yan et al. isolated MSCs from human HCC tissues and demonstrated that the co-culture of these MSCs with HCC cells enhanced tumor formation and increased liver and lung metastasis. Tumor-associated MSCs produced several trophic factors including S100A4 that upregulated miR-155, leading to HCC proliferation and invasion (90). Similar data from Hernanda et al. indicated that conditioned medium from MSCs isolated from HCC tissues had trophic effects on the Huh7 hepatoma cell line in vitro and in vivo (91). It was also demonstrated that HCC-associated MSCs promoted EMT and liver tumorigenesis through the expression of a lncRNA-MUF (MSC-upregulated factor) in HCC tissue (92). These data suggest that MSCs can be educated by the tumor to favor its own growth. However, due to the heterogeneity of MSCs, and therefore the difficulty to investigate the endogenous MSCs, more studies are necessary to establish the precise role of these cells on tumor development.

The persistent inflammatory milieu not only promotes tumor development but also accelerates tumor progression, stimulates the formation of new blood

vessels, and remodels the ECM. Thus, TAMs are also considered as crucial players in tumor progression. In HCC, TAMs stimulate invasion, angiogenesis, and metastasis through the release of several mediators, including IL-6, IL-8, TNFα, TGFβ, EGF, VEGF, MMP-2, and MMP-9(93). These factors also promote EMT, which is a crucial event for tumor progression and metastasis (18, 22, 23, 37). In addition, infiltrating monocytes in HCC express high levels of programmed cell death-ligand 1 (PD-L1) that binds to PD-1 on $CD8^+$ T cells, suppressing its anti-tumoral cytotoxic activity (94).

The interaction of HA with its main receptor, CD44, promotes tumoral signaling involved in cell proliferation, invasion, chemoresistance, EMT, and angiogenesis (23). Hepatic HA accumulation may be linked to increased tumor tissue stiffness (95), which is associated with HCC development. HA was demonstrated to facilitate the aggressive phenotype of HCC cell lines, promoting cell proliferation, metastatic potential, and aerobic glycolysis switch in MHCC97H and HepG2 cells, both in vitro and in vivo (96).

PGs can regulate the bioavailability and activity of hormones, growth factors, cytokines, and their respective receptors which in turn can affect gene expression, tumor phenotype, tumor progression, and recurrence rates in specific tumor types (97). During angiogenesis, decorin induces endothelial cell sprouting and activates intracellular signal transduction pathways. Decorin interacts with several angiogenic growth factors, including VEGF, platelet-derived growth factor, fibroblast growth factor, IGF, connective tissue growth factor, and hepatocyte growth factor (98). In addition, decorin interacts with TGF-β and neutralizes its activity, preventing the binding to its receptor, and therefore plays a significant role in tumor progression and angiogenesis (67). Decorin can also play a pro-angiogenic role by facilitating endothelial cell adhesion and migration on type I collagen (99).

TARGETING THE MICROENVIRONMENT TO INHIBIT TUMOR GROWTH

TAM-targeted therapies are usually aimed at: (i) eliminating TAMs, (ii) blocking the recruitment of circulating monocytes, and/or (iii) reprograming TAMs to an anti-tumor phenotype. For example, it was reported that in mouse models of HCC, treatment with the tyrosine kinase inhibitor sorafenib reprogrammed TAMs and promoted the stimulatory activity of hepatic NK cells (100). Zoledronic acid was demonstrated to have an anti-tumor effect by targeting TAMs through phagocytosis by macrophages and induction of apoptosis (101). The therapy combining these two drugs, sorafenib and zoledronic acid, is currently being evaluated for the treatment of advanced HCC in phase II clinical trials (NCT01259193). Another strategy for targeting TAMs is inhibition of glypican-3, a proteoglycan that promotes the recruitment of macrophages into the tumor, by specific antibody (102). This strategy is currently in phase I clinical trials for advanced HCC (103). In addition, there are two more trials (NCT02723942 and NCT02395250) that use a similar strategy. So far, the most critical issue that TAM-targeted therapies need to overcome is the need to repolarize macrophages towards an anti-tumor behavior without causing any adverse events.

The abnormal metabolism of HA and its accumulation in the injured liver or an established tumor have led to the consideration that inhibition of HA synthesis may avoid tumor progression and metastasis. Several reports propose the use of 4-MU as an inhibitor of HA synthesis or the targeting of its receptor CD44 as anticancer treatments. The use of CD44 antisense oligonucleotide increased chemosensitivity to doxorubicin significantly and induced apoptosis and necrosis in HCC cell lines (104). The treatment of HCC cells with 4-MU significantly reduced tumor cell proliferation and induced apoptosis, without affecting normal hepatocytes. Systemic treatment with 4-MU resulted in the induction of necrosis and reduction in the number of tumor satellites in an orthotopic fibrosis/HCC mouse model. Mice treated with 4-MU had reduced levels of fibrosis and decreased the number of activated HSCs when compared with controls (18). This antitumor property could be associated with an inhibition of angiogenesis and decrease in IL-6 production (19). Furthermore, animal survival was increased when $CD133^{low}$ HCC cells, generated upon 4-MU treatment, were injected in a metastatic HCC model (105).

There is clear evidence that PG composition changes with liver cancer development. Thus, it could constitute targets for potential therapeutic agents and diagnostic biomarkers. Decorin represents a powerful tumor cell growth and migration inhibitor by modulating both tumor stroma deposition and cell signaling pathways (106). Soluble decorin acts as a tumor suppressor mainly by downregulating various receptor tyrosine kinases (such as EGFR, Met, IGFR, and VEGFR), β-catenin, and Myc expression, and upregulating p21WAF1/CIP1 (106, 107).

CONCLUSION

The HCC microenvironment is composed of several tumoral and non-tumoral cell types, and ECM components that are in continuous communication and interaction with each other. The cellular components include CSCs, LPCs, MSCs, and various populations of immune cells including TAMs. The major ECM components that are altered in HCC are GAGs such as hyaluronan, and PGs including decorin and syndecan. Their interactions make an important contribution to tumor progression by modulating tumor cell properties. The data generated in preclinical models and clinical trials targeting the TME, especially these molecules and cell types, show highly promising results; however, their clinical utility is yet to be ascertained. In addition, adverse events of such therapies need to be cautiously evaluated. A better knowledge of the microenvironment–tumor cell interactions could be useful and beneficial for the development of new therapeutic approaches for HCC.

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3

In Vitro Models of the Liver: Disease Modeling, Drug Discovery and Clinical Applications

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Abstract: In vitro models of the liver have led to important insights into the pathogenesis of liver disease. These models are essential tools in the discovery and preclinical stages of drug development. The clinical application of these models is also emerging as a promising avenue for validating genetic target-matched treatments, in a precision medicine approach to treatment. Recent advances in 'liver-on-a-chip' technology and liver organoid research have opened up new opportunities for the functional and clinical use of organotypic in vitro models. This chapter focuses on the currently available in vitro liver models and the opportunities and limitations they present in the context of evaluating their use in disease modeling, drug discovery, and clinical application.

Keywords: liver-on-a-chip; organotypic cultures; precision-cut tissue slices; sandwich-cultured hepatocytes; whole organ explants.

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INTRODUCTION

The large burden of liver disease and primary liver cancer along with the management difficulties encountered have provided the impetus to pursue the use of representative in vitro models of liver function, response to injury, and development of malignancy. Improved 2D and 3D in vitro disease models would enhance our understanding of the cause of liver injury and cancer, increase the efficacy of preclinical drug discovery, and be a useful clinical tool for precision medicine. The increasing popularity of organ-on-a-chip technology and improvements in 3D cell cultures has enabled unique insights into liver disease (1, 2). This chapter focuses on the current types of in vitro liver models, the opportunities and limitations of their uses in drug discovery, basic research and clinical management, as well as new directions of this field.

IN VITRO LIVER MODELS

In this overview, in vitro models will be defined as the culturing of isolated tissue components of an organ, while preserving many aspects of the in vivo environment. The chief purpose of in vitro models in research and medicine is to minimalize experimental variables to effectively isolate different organ components or structures for study under well-controlled, reproducible, and easily assessed conditions. This overview will focus on 2D and 3D models of the liver based on organotypic characteristics, including cell type, liver function, and zonation, and likely application in basic research, drug discovery, and clinical practice (Figure 1).

The liver has a heterogeneous cellular composition that includes hepatocytes (the target of most disease and comprise the majority of the liver in quantity and volume), Kupffer cells (liver-resident macrophages), hepatic stellate cells, liver sinusoidal endothelial cells (LSECs), biliary epithelial cells, fibroblasts, immune cells, and adult stem cells. Important liver functions to consider include: (i) the metabolism of endogenous substrates and exogenous compounds; (ii) the regulation of amino acids, carbohydrates, and fatty acids; (iii) synthesis of proteins (such as albumin or transferrin) and bile synthesis; (iv) immune activation upon injury; (v) the biotransformation of xenobiotics; and (v) the resilience to senescence (1, 2). The cytochrome P450 (CYP450) family of abundant enzymes is also significantly important to liver function as they mediate the metabolism of drugs (3).

The lobules of the liver are complex with perivenous, intermediary, and periportal zones (4). The intercellular oxygen concentration of the lobule is 15–20 mm Hg in the perivenous zone compared to 45–50 mm Hg in the periportal zone (5). Metabolic processes, including glucose uptake, glycolysis, amino acid synthesis, bile acid production, and glucuronidation, are all greater in perivenous cells, which also have the greater CYP450 enzyme activity. By contrast, oxygen uptake, glucose delivery, gluconeogenesis, urea synthesis, fatty acid oxidation, cholesterol synthesis, and sulfation are all comparatively greater in periportal cells. Non-parenchymal cells such as bile duct cells and hepatic stellate cells are more abundant in the oxygen-rich periportal zone. These observations are important to liver modeling

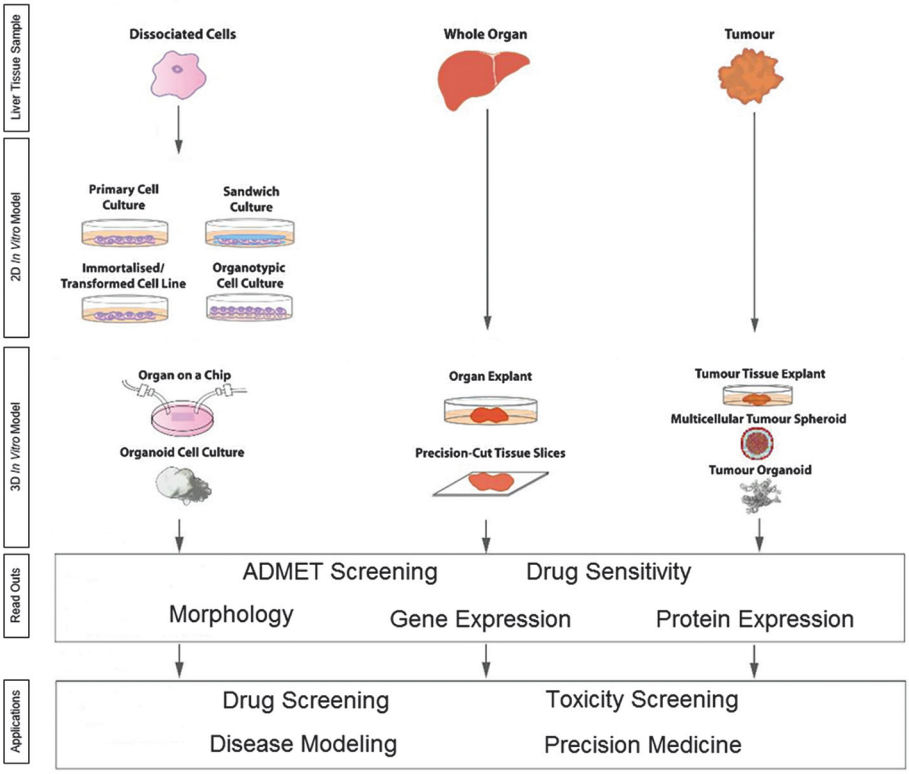

Figure 1 Overview of 2D and 3D in vitro models of the liver. Flow diagram indicates the in vitro models of the liver, their readouts, and applications. Each model was categorized by the type of sample it is derived from and whether it is 2D or 3D model.

as zonation is important and disrupted in liver diseases, especially diseases associated with hypoxia and reactive oxygen species like non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (HCC) (6).

Increasing the efficacy of drug development and toxicity testing by improving in vitro models is of great interest to researchers and the pharmaceutical industry (7). In 2015, the cost of bringing a new drug to market was estimated at 2.6 billion USD (8), with the major contributor to this cost being the very low clinical success rate of new compounds (approx. 11.8%) (9). This high burden of cost necessitates the exploration of new approaches, including advances in preclinical methods, which select new drug interventions for clinical trials.

In the discovery and preclinical development stages of drug development, candidates are identified by correlating drug responses in cell cultures and preclinical animal models—usually one rodent and one non-rodent species (10). Screening for absorption, distribution, metabolism, excretion, and toxicity (ADMET screening, also commonly referred to as ADME or the study of drug metabolism and pharmacokinetics) optimizes preclinical testing by enabling better understanding of the pharmacokinetic and pharmacodynamic properties of drug candidates (11).

Desirable drug-like properties identified by ADMET screening include adequate absorption and distribution, low metabolism, complete elimination from the body, and a minimal toxicological risk (10).

A significant challenge in this field is predicting human-specific liver toxicity (12). Animal models do not always reflect human toxicity due to differences in physiology, interspecies metabolic capacities, and disease adaptations. Similarly, in vitro models often do not accurately predict toxicity due to non-linear dose–toxicity relationships, unclear mechanisms, non-organ-specific toxicity, as well as adverse downstream effects (1, 12). Drug-induced hepatic injury is the most frequently cited reason for approved drugs being removed from the market (13). Current 2D in vitro assays are based on cell lines such as HepG2 that have reduced metabolic capacities compared to primary hepatocytes, while the use of primary human hepatocytes suffers from high donor-to-donor variation and cultures only retain in vivo characteristics for a short time ex vivo (11). The effect of improving these in vitro assays may potentially lead to more effective and rapid pre-clinical drug development.

After the completion of the human genome project in the early 2000s, there was significant optimism for the potential of genomic medicine to revolutionize the diagnosis and treatment of many illnesses, in particular, the clinical application of genetic predictors to better understand patient risks of disease and responsiveness to potential designer drugs, based on targeting specific molecular pathways (14). In 2011, the US National Research Council coined the term "precision medicine" to inspire a new taxonomy for disease via a knowledge network. They defined precision medicine as

> "The tailoring of medical treatment to the individual characteristics of each patient […] to classify individuals into subpopulations that differ in their susceptibility to a particular disease or their response to a specific treatment. Preventative or therapeutic interventions can then be concentrated on those who will benefit, sparing expense and side effects for those who will not. This is different from personalized medicine, which refers to 'an approach to patients that considers their genetic make-up but with attention to their preferences, beliefs, attitudes, knowledge and social context'." (15)

The disease treatment strategies that have so far benefited the most from precision medicine are treatments for cystic fibrosis and cancer management using genome sequencing to enhance patient care by improved diagnostic sensitivity, allowing for more precise genetic therapeutic targeting (16). Since the early success of the Bcr-Abl kinase inhibitor Imatinib for targeted therapy for chronic myeloid leukemia, oncology has moved towards molecular classification (16), but currently there are only 11 genomic alterations known to drive tumor progression in different tissues matched directly with approved targeted therapies (17).

CONVENTIONAL 2D IN VITRO LIVER CELL CULTURES

Essentially, cell biology relies on 2D models generated from dissociated cell cultures that are expanded on plastic surfaces, often supported by extracellular matrix (ECM) scaffolding. These are primary cell cultures derived directly from

harvested tissue or immortalized cell lines (primary cells genetically transformed to produce rapidly proliferating, uniform, easily cultured, artificial phenotypes). A major reason for the popularity of dissociated cell cultures is that the majority of mammalian cells can be expanded into adherent colonies on culture plates, and these have proven to be relatively low cost and easy to manipulate and maintain. A high-throughput cultured monolayer of cells receives a consistently homogenous amount of nutrients, growth factors, and exposure to oxygen. Commercialized cell lines are available across a diverse range of tissue types, and there is extensive commercial support for these cultures, such as the availability of different culture media and consumables. Furthermore, there are various options for genetic manipulation, such as CRISPR, gene transfer, insertion, deletion, silencing, and cell fusion (1).

Primary cell cultures

Human hepatocyte primary cell cultures are a physiologically relevant model for studying drug biotransformation and toxicity (18, 19). However, cells grown in this way have a number of issues. They only maintain their wild-type characteristics for a limited time when cultured on 2D surfaces because of de-differentiation. In addition, in vitro manipulation often results in a loss of wild-type characteristics, slow proliferation, changes in metabolism, and early senescence after a limited number of passages (18, 19). Therefore, cell cultures require successive tissue harvests, which incur higher associated costs. Moreover, the harvesting of tissue is susceptible to contamination from non-applicable cell types, thereby compromising the model's integrity (1).

The ECM has a profound effect on primary cell function, differentiation, signaling, and morphology (20, 21). For example, culturing primary hepatocytes with the scaffold matrix Matrigel® induces gene expression, which more closely resembles liver tissue in vivo. It also improves cellular morphology by enhancing cuboidal shape and results in cells with clearly defined cell borders that allow the formation of highly organized cellular networks (22).

Primary hepatocyte cell cultures have been useful for understanding the mechanisms in liver regeneration (23) and for discerning the relationship between the liver cytoskeleton and liver-specific protein expression (24). Similarly, primary cultures of hepatic stellate cells have been instrumental in understanding the causes of liver fibrosis and identification of key fibrogenic mediators (25, 26). In drug testing, primary human hepatocyte cell cultures are considered the "gold standard" because they display many phenotypic functions of the liver when compared to other in vitro models (27, 28). However, this approach has been heavily criticized as suboptimal. The common issues include: (i) cells being cultured at densities of only approximately 1% of physiologically normal tissue densities, thereby impairing intercellular signaling; and (ii) cultures being non-homeostatic as conditions are optimized for rapid growth, thereby preventing correct cell differentiation (29, 30). Primary hepatocytes experience a decline in CYP450 expression when grown in vitro (31), while the transcription of common genes is unaffected leading to a decrease in CYP450 proteins and activity, significantly limiting the translatability of this model (32).

Sandwich-cultured hepatocytes

Culturing primary hepatocytes between two layers of collagen, termed sandwich-cultured hepatocytes (SCH), results in retained cellular polarity with correct localization of basolateral and canalicular transporters as well as formation of functional bile networks (33, 34). Discovered by Dunn and colleagues, SCH maintain mRNA expression, as well as cell functions, such as the secretion of albumin, transferrin, fibrinogen, bile acids, and urea for 6 weeks (35, 36), and CYP450 isozymes for 2 weeks (37). SCH have proven to be a useful tool to study hepatobiliary drug disposition and mechanisms of drug-induced liver injury, for example, elucidating transport mechanisms responsible for the elimination of the antifungal agent, micafungin (38), and the mechanisms of bile acid-mediated, drug-induced liver injury (39).

Immortalized or transformed cell lines

Immortalized or transformed cell lines are dissociated cell cultures, which have been genetically modified or selected for an oncogenic phenotype. Typically, these cultures show rapid proliferation, resistance to de-differentiation, improved passaging, and greater resilience to senescence, making these cells convenient to maintain, expand, and retain phenotypic consistency between experiments. These cell cultures have been successfully used to study hepatitis B virus (HBV) and hepatitis D virus (HDV) infections. Mechanisms of HBV viral entry were discovered in HepRG cell lines (40), the expression/replication of HBV was discovered in HepG2 (41), and the complete HDV replication cell cycle was discovered in HepG2 and Huh7 cells (42, 43). The shortcomings of these cell lines include significant changes in differentiation potential; altered genomic content (44); abnormal proteome expression; and the loss of features such as cellular polarity (45), contact inhibition (46, 47), metabolic CYP450 potential (48, 49), the induction of inflammatory mediators (50), as well as paracellular transport (51).

Due to most immortalized human hepatic cell lines having reduced liver-specific functionality (52), different strategies have been used to counteract this issue, including co-culture systems with primary human hepatocytes and overexpressing liver-enriched transcription factors, CYP450 enzymes, or proliferation inhibitors to increase hepatic functions (52). Immortalized human hepatic cell lines have been successfully used to investigate the life cycle of hepatitis C and B viruses (53–56), and they act as cellular models of hepatocarcinogenesis (57) and steatosis (58). Furthermore, immortalized hepatic cell lines have also been found suitable as in vitro tools for drug screening and safety testing. Hc3716-hTERT, immortalized fetal hepatocytes, and telomerase-immortalized hepatic stellate cells NPC-hTERT have been used as models for predicting the side effects of telomere-targeting drugs (59), and Fa2N4 cells have been used for screening pregnane X receptor-mediated CYP3A4 induction (52).

Organotypic cultures

A major limitation of dissociated cell cultures is their high degree of homogeneity as they fail to represent liver tissue heterogeneity. While hepatocytes comprise the

majority of cells within the liver, liver function is dependent on a number of different cell types. 2D organotypic culture uses multiple different cell types to recapitulate in vivo-like cell heterogeneity. Co-cultures of hepatocytes and macrophages have been successfully used to model their intercellular cross-talk, their roles in the regulation of liver regeneration, hepatocyte function, and the acute-phase response to septic liver injury (60). Long-term co-cultures of hepatocytes and LSECs, either on top of or sandwiched between a collagen gel, retained the LSEC phenotype and enhanced hepatocyte functions, such as increased CYP450 activity (61). In contrast, co-cultures of primary hepatocytes and endothelial cells, maintained under high oxygen conditions, preserved cell morphology, high CYP450 levels, and native gene expression (62). A recent example by Ware and colleagues was a triculture of primary human hepatocytes with 3T3-J2 fibroblasts and LSECs overlayed with Matrigel®, which was shown to display a stable phenotype with increased albumin and urea secretion for 3 weeks (63).

Shortcomings of conventional 2D liver cell cultures

While these models have many benefits, a significant issue with 2D liver models is their lack of hepatic sinusoid heterogeneity, in vivo-like cell density, oxygen-induced zonation, and the liver circulatory system. The clinical application of 2D cell cultures is limited due to significant issues of cell contamination, non-reflective cell differentiation, genetic drift, variable drug responsiveness, and a limited capacity to predict toxicity, creating a degree of uncertainty when using 2D culture as a model for potential treatments, with a possible exception of patient-derived tumor cell lines for precision medicine (64).

3D IN VITRO LIVER MODELS

The shortcomings of 2D cell culture models have driven the development of 3Dcell culture techniques. The advantages of 3D models include replicating the complex attributes of the liver beyond liver-specific metabolism, such as increased cell density, organization, and cell–cell signaling, O_2 zonation, as well as the anatomy of the liver lobule and the circulatory system (Figure 2). Some of these models are limited by their low applicability for high-throughput screening as well as their laborious preparation, lack of reliable protocols, and short-term survival of these models in culture. However, 3D models have proven useful in developmental and toxicological studies and represent an exciting opportunity for more functionally relevant clinical modeling.

Whole organ explants

Whole mouse liver organ explants have been used to study the effects of oxidation on the progression of hepatocarcinoma. In 2016, Torricelli and colleagues reported inoculating the murine hepatocarcinoma cell line Hepa 1/A1s into the livers of live mice, which proliferated in vivo for 20 days before the livers were removed and used as a whole organ explant model to study the effects of the antioxidant Citozym on tumor size in culture over a 4-week period (65).

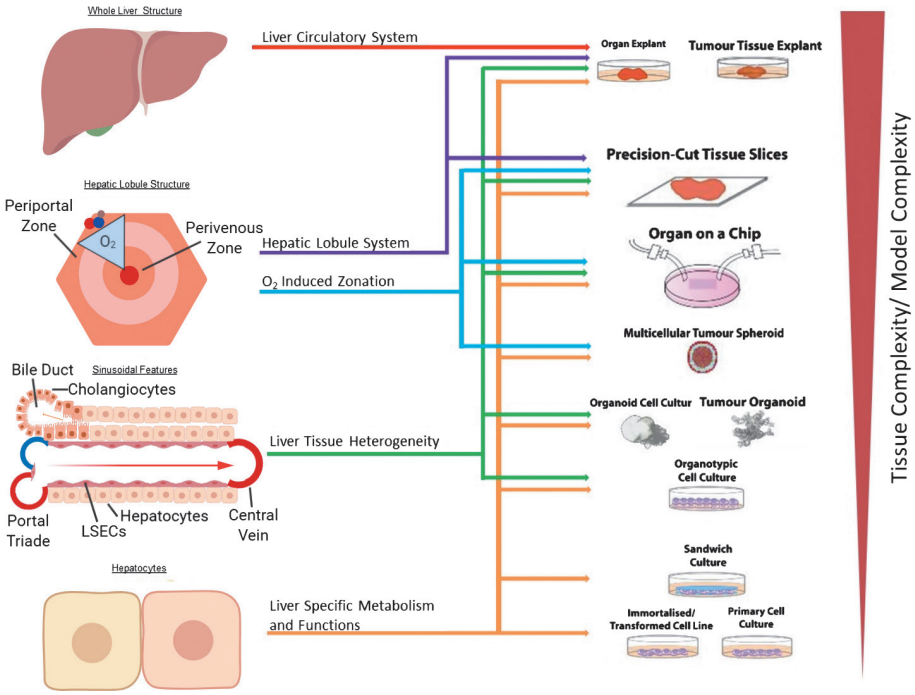

Figure 2 Different levels of structural complexity in the liver and their attributes represented in in vitro models. The different structures of the liver and their corresponding liver models on a gradient, based on their tissue complexity. Structures of the liver are then linked by attributes represented in the in vitro models discussed in this chapter.

Precision-cut tissue slices

Liver 'precision-cut tissue slices' (PCTS) have mostly been generated using rat livers, but the technique has also been used for other species including humans (66). Slicing allows sufficient oxygen and nutrient supply to the inner cell layers, and hepatocytes retain their membrane and intracellular polarization (67). In a study by Vickers and colleagues, rat liver slices have been found to be fully capable of metabolizing compounds and maintaining fibrogenic pathways, such as activation of stellate cells, the proliferation of myofibroblast-like cells, and an increased collagen deposition for4 days under appropriate conditions (68). As with 2D cultures, CYP450 expression decreases during prolonged culturing, but this has been shown to slow down when the medium is supplemented with insulin, dexamethasone, and fetal calf serum (69, 66).

Tumor tissue explants

A 'tumor tissue explant' is a 3D model of cancer, where an excised human tumor is embedded in collagen and tissue culture medium (70). Mainly used as an in vitro model of drug efficacy, this method has been demonstrated by Vaira and

colleagues to preserve pathway activation, pharmacological inhibition, internal 3D architecture, cell viability, and global gene expression profiles up to 5 days ex vivo (71). Unfortunately, this model is relatively unreproducible due to tissue heterogeneity, applicability of techniques such as imaging and flow cytometry is limited, and the culture is only viable for a short period of time, making it impractical for any form of high-throughput, long-term, or clinical investigations (72).

Multicellular tumor spheroid

The best-characterized 3D organotypic models of cancer are "multicellular tumor spheroids," which are constructed from homogeneous tumor cells or co-cultures on nonadherent surfaces, where the cell suspension undergoes aggregation and compaction (73, 72). Spheroids re-establish morphological, functional, and physiological cellular transport properties of their corresponding tissue and resemble the avascular tumor nodules/micrometastases or intervascular regions of large solid tumors (74). These have been used to gain insights into therapeutic challenges associated with drug resistance, metabolic and proliferation gradients, and the importance of cell–cell/cell–matrix interactions (74). Liver multicellular tumor spheroids have been used for understanding microenvironmental chemoresistance of HCC associated with the crosstalk between HCC cells, hepatic stellate cells and other stromal cells (75, 76). For instance, liver cancer spheroids of Huh7 cells co-cultured with human umbilical vein endothelial cells promoted HCC gene expression and oncogenic properties, such as cell proliferation, increased expression of cancer stem cell markers, and extracellular cytokine-mediated signaling (77). Furthermore, this multicellular tumor spheroid model tolerated higher anti-cancer drug concentrations than the monolayer control, which may be due to the hypoxic conditions within the spheroid, activating extracellular signal-regulated kinases (ERK), critical in tumor cell proliferation (77).

Organ-on-a-chip

An "organ-on-a-chip" utilizes the microfluidic technology to replicate the in vivo microenvironment and homeostasis of living human organs (78). Often consisting of transparent 3D polymeric microchannels lined by human tissue cultures, these devices are designed to mimic the 3D microarchitecture, organ-specific mechanical/biochemical microenvironment, and the functional tissue–tissue interfaces in organs. Many investigators use micro-channels of matrix-coated porous membranes with a layer of endothelial cells, populated by the desired co-culture, connected by wells containing the preferred perfusion medium (78). These devices have also been designed with compartmentalized channels, allowing for independent fluidic/aerosol access to individual tissue types, enabling selective treatment conditions and analysis (73). "Liver-on-a-chip" systems have been shown to predict clearances, toxicity, and the mechanism of action of certain drugs (79).

Commercially available liver-on-a-chip microfluidic systems such as the 3D perfused cell culture platform from Zyoxel and the Microliver chip from HμRel® corporation have been used for toxicity testing, but none of these systems have been fully validated. Most current models use primary human hepatocytes to populate the system, and a few include a co-culture with non-parenchymal cells,

which has improved their capacity to predict liver toxicity (80). For example, the anticancer prodrug Flutamide was tested for hepatotoxicity using human HepG2/C3a cells in a microfluidic biochip and led to metabolic results consistent with reports in the literature. The authors demonstrated perturbation of the tricarboxylic acid cycle and impaired urea cycle with reduced uptake of essential amino acids (81). In 2016, Bhise and colleagues have also had success in drug toxicity analysis with a liver-on-a-chip platform using human HepG2/C3a spheroids encased in hydrogel within a bioreactor for long-term culturing (82). Furthermore, biochips using primary hepatocytes have been used to measure the pharmacokinetics of several drugs, with results that resemble data in relevant clinical trials (83). The use of human-induced pluripotent stem cells (hiPSCs) to generate hepatocyte-like cells has been assessed for populating liver-on-a-chip systems. However, differentiated cells were found to have reduced functionality and immature gene/protein expression (84). Focused efforts at recapitulating lobule zonation using liver-on-a-chip systems have had mixed success (85, 86). A controlled oxygen gradient has been maintained in primary rat hepatocytes, which induced in vivo-like heterogeneous CYP450 localization and toxicity. This is significant because most studies only model one lobule zone (usually the perivenous zone), and hence, the expression of intermediate metabolites may be exaggerated, while detoxification may be underestimated (79).

Organoids

An "organoid cell culture" is defined as a collection of several cell types that develop from stem cells or organ progenitors, display organ-specific functions, mimic its structural organization, and self-organize through cell sorting and spatially restricted lineage commitment, similar to organogenesis in vivo (87). Organoids are usually formed by exploiting the expansion potential of three cell types: pluripotent embryonic stem (ES) cells, induced pluripotent stem cells (iPSC), or organ-specific adult stem cells (aSC), forming new primary tissue buds, made of self-organizing daughter cells that are induced to differentiate in culture. These daughter cells display the capacity to self-organize into structures that reflect crucial aspects of the tissue for which they are fated (88). What distinguishes 3D liver organoid cell cultures from other in vitro models is that they bridge the gap between the microenvironmental integrity of organ explants and PCTS, yet have the high-throughput accessibility of immortal cell lines.

Liver organoids have demonstrated advantages over conventional in vitro models such as long-term genetic stability, in vivo-like organization, and maintaining the necessary cellular crosstalk and behavioral characteristics of their primary corresponding cells (89). For example, adult stem cells from alpha-1 antitrypsin (A1AT)-deficient patients cultured into liver organoids mimic the in vivo situation with A1AT protein aggregates and signs of endoplasmic reticulum stress (89). Liver organoids were first created by Huch and colleagues by exploiting the expansion potential of $LGR5^+$ progenitor/stem cells in damaged adult mouse liver tissue, by Wnt-driven regeneration. They then induced hepatocyte maturation by inhibiting Notch and TGF-β signaling, which led to the expression of genes involved in cholesterol and lipid metabolism, as well as from the CYP450 superfamily. Immunofluorescent analysis revealed the expression of hepatocyte

nuclear factor 4α and albumin, hepatocyte binucleation, as well as patches of the progenitor cell and ductal marker cytokeratin 19. Ninety percent of these cells were also competent for low-density lipoprotein uptake and accumulated glycogen (90).

In addition, Huch and colleagues established the first organoid culture system for human liver from primary bile duct stem cells (89). These organoids displayed high stability, both chromosomally and structurally, with low rates of genetic alterations over a 3-month culture. Using the established methods developed for mouse liver organoids, they induced hepatocyte differentiation in the human liver organoids. As a consequence, the cultures began to display hepatocyte gene expression, albumin secretion, CYP450 metabolism, bile acid production, ammonia elimination, low-density lipoprotein uptake, and glycogen storage (89). Further, organoids were readily engrafted in vivo upon transplantation in mice (89).

It has been proposed that liver organoids may be a useful model for studying the transition of NAFLD to non-alcoholic steatohepatitis if these organoids were co-cultured with hepatic stellate cells, Kupffer cells, and other inflammatory cells (91). Retroviral transduction and liposomal transfection have been successfully used to genetically manipulate liver organoids with green florescent protein-expressing vectors (92). Another exciting avenue to explore is CRISPR gene editing, with success already achieved using intestinal organoids of cystic fibrosis patients, where the cystic fibrosis transmembrane conductance regulator (*CFTR*) locus was corrected in vitro by homologous recombination (93).

Although 3D liver organoid cell cultures are becoming a research focus, challenges for the technology include the recapitulation of the in vivo ECM. It has been suggested that the use of decellularized liver ECM populated with liver organoids may improve hepatocyte functions (89), which has had success in promoting survival and maturation compared to collagen type I (94). Limitations of liver organoids include the lack of a native microenvironment, thus inhibiting the study of the interactions between stem cells and their niches, a lack of all necessary in vivo growth factors or signaling gradients, and an inability to accurately model immune responses. A possible solution to this is organotypical co-culturing and the application of microfluidic technologies. Further heterogeneity between organoid cultures can cause inconsistency in reproducing phenotypic traits such as size, shape, cellular composition, and 3D architecture (95, 96).

In drug development, an in vitro organoid system comprised of human cells which are complex enough to demonstrate organotypic composition, morphology, and functionality (Table 1) would be ideal in closing the gap in phenotypic drug discovery (26). Increasing the chain of translatability for target-agnostic investigations remains a significant challenge (3), and human organoids may build a rational and sustainable discovery pipeline, reducing false-positives and cost. The reason for this is that organoids may present a more phenotypical disease-associated functional response to treatment than 2D cell lines as well as a more accurate disease-free associated phenotype. Phenotypic drug discovery with generic readouts like viability or apoptosis in cancer cell lines often provides little insight into disease pathways or mechanisms of action, while in vitro 3D organoid models exhibit the potential to become highly predictive cell-based tools for preclinical drug toxicity assessments (97).

TABLE 1 **Assessment of in vitro liver models based on their organotypic morphology/functions/interactions, technical ease of use, ease of maintenance, and throughput, with comment on their benefits and disadvantages. Each category is graded either as good (green), moderate (yellow) or poor (red)**

	Primary Cell Culture	Sandwich Culture	Immortalised/ Transformed Cell Line	Organotypic Cell Culture	Organ Explant	Precision-Cut Tissue Slices	Tumour Tissue Explant	Multicellular Tumor Spheroid	Organ on a Chip	Organoid Cell Culture	Tumour Organoid
Organotypic Morphology/ Function/ Interactions											
Technical Ease of Use											
Ease of Maintenance											
Throughput											
Comments	Hepatocytes Preserve Many Phenotypic Functions Low Cost (Tissue Dependent) Extensive Commercial Support De-Differentiate within a Short Time No ECM Unnatural Gene Expression/ Morphology Impaired Cell Signalling Decline in CYP450 Expression Susceptible to Contamination No Zonation	Hepatocytes Preserve Many Phenotypic Functions for 6 Weeks Retain Cellular Polarity Low Cost Present ECM No Zonation	Rapid Proliferation Low Cost Extensive Commercial Support Resistant to De-Differentiation Phenotypically Consistent Wide Variety of Commercially Available Genetic Variants Easy to Genetically Manipulate No ECM Impaired Cell Signalling Changed Differentiation Potential Altered Genomic Content Abnormal Proteome Loss of Cellular Polarity Decline in CYP450 Expression (Line Depended) No Zonation	Preserves Hepatocyte and Non-Parenchymal Cell Signalling, Preserving Some Functions Low Cost (Tissue Dependent) May Contain ECM No Zonation	Preserves Hepatocyte and Non-Parenchymal Cell Signalling, Preserving Some Functions Low Cost (Tissue Dependent) May Contain ECM No Zonation	Preserves All In Vivo Structures and Functions Sufficient Oxygen and Nutrient Supply Laborious Preparation and Maintenance Decline in CYP450 Expression	Preserves All In Vivo Structures and Functions Short-term Survival Laborious Preparation and maintenance Unreproducible	Re-establishes Morphological, Functional and Mass Transport Properties Preserves Cellular Cross-Talk, Proliferation and Drug-Resistance Present ECM Zonation Present	Preserves Hepatocyte and Non-Parenchymal Cell Signalling, Preserving Some Functions Preserves Homeostasis Sufficient Oxygen and Nutrient Supply Large Range of Different Designs May Contain ECM (Design Depended) Zonation Present (Design Depended)	Re-established In Vivo Cell Organisation, Morphology, Microenvironment and Function Preserves Cellular Cross-Talk, Proliferation and Drug-Resistance Long-term Genetic Stability Present ECM High Heterogeneity No Zonation	Re-established In Vivo Cell Organisation, Morphology, Microenvironment and Function Preserves Cellular Cross-Talk, Proliferation and Drug-Resistance Long-term Genetic Stability Present ECM High Heterogeneity No Zonation

Good Moderate Poor

To date, there have only been a few successful clinical uses of in vitro organoid models. One example was a robust functional drug assay for cystic fibrosis, developed using human intestinal organoids, which demonstrated the clinical potential organoids hold for precision medicine (98). Using automated fluorescent image analysis, the function of the CFTR (which is defective in cystic fibrosis) can be assessed, allowing the authors to efficiently test drug responses of patients and treat rare forms of this disease (99). This assay has advantages over established in vitro models, such as rectal biopsies and primary airway tissue culture models because organoids can be passed into large screening arrays for high-throughput precision medicine (98).

Tumor organoids

Despite the precision medicine approach, only a minority of patients with cancer derive clear benefit from matching genetic targets with treatment. Currently, precision oncology based on emerging biomarkers remains an investigational strategy, and the present approach of matching single agents to patients is still suboptimal (17).

To address this issue, Pauli and colleagues piloted a study that combined whole-exome sequencing (WES) of patient metastatic and primary tumors with tumor organoid drug sensitivity assays, facilitating the integration of genomic data with drug screening in an iterative platform to identify effective therapeutic regimens for individual patients (100). Sequencing of 769 specimens identified somatic cancer gene alterations that were actionable by FDA-approved drugs in three specimens (0.4% of the total), but found somatic alterations with potential clinically actionable by off-label use in 71 of the remaining specimens (9.6% of the total). Fifty-six organoid tumor lines and 19 patient-derived organoid xenografts were successfully established and characterized using cytology and histology, leading to patient-derived tumor organoids from four candidates being selected and subjected to 2D high-throughput drug screening. The tumors screened were from uterine carcinoma, endometrial carcinoma, and two lines of stage IV colorectal cancer. Single and combination compounds selected by this process were then validated in 3D cell culture. Drug combinations were further validated in patient-derived xenografts for two patients. In both cases, the drugs selected by the screening were found to be more effective at reducing tumor growth than the patient's current regimen. These results demonstrate that the optimal drug combinations can be identified using sequential drug-sensitivity screens followed by validation in personalized patient-derived tumor organoid xenograft models in a clinically relevant timeframe of 7 and 12 weeks (100). The further utility of tumor organoids to be passaged for large data sets while retaining individual phenotypic characteristics cannot be under-appreciated, as the power to rationally delineate the optimal therapy for every individual patient removes ambiguity and could exponentially speed up the rate of patient recovery. Pauli and colleagues demonstrated that 3D patient-derived tumor organoids can be a powerful tool for individual drug sensitivity assays, the results of which can be verified in xenograft models (100). However, these findings still need to be proven in clinical practice and shown to benefit patient outcomes.

In late 2017, the clinical potential of tumor organoids derived from human primary liver cancer was demonstrated by Broutier and colleagues (101). Tumor organoids from HCC, cholangiocarcinoma (CC), and combined hepatocellular-cholangiocarcinoma (CHC) retained features from their tissue of origin, such as the vast majority of cancer-related genetic variants, gene expression profiles, and tissue histologies. Immunohistochemistry and immunofluorescence showed that even after long-term expansion in culture, disease-specific protein expression was conserved, including the HCC markers HepPar1 and alpha-fetoprotein in HCC/CHC, and the ductal/CC marker epithelial cell adhesion molecule (EpCAM) in CC/CHC. Once established, the liver tumor organoid cultures were used to develop drug assays to identify patient-specific drug sensitivity. This was achieved by using a simple cell viability assay and observing the rate of organoid viability in the presence of range treatments with drugs such as sorafenib, gemcitabine, and SCH772984. Of these drugs, the sensitivity of the ERK inhibitor SCH772984 was then able to be validated in a patient-derived xenograft model transplanted with a CC tumor organoid (101).

Another precision medicine study by Nuciforo and colleagues, using human liver tumor organoids, found that HCC tumor-derived organoids maintained the growth pattern and differentiation grade of the originating primary tumor. In addition, alpha-fetoprotein, glypican 3, glutamine synthetase, and heat shock protein 70 protein expressions were preserved. Whole exome sequencing determined that somatic and non-synonymous somatic mutations in the HCC biopsies were observed at a rate of 88 and 90%, respectively, in the corresponding HCC organoids at early passages. The tumor organoid cultures also displayed variable sensitivity to sorafenib exposure demonstrating that organoids derived from biopsies can be used to test tumor-specific sensitivities to growth-inhibitory substances. However, a direct comparison of in vitro sorafenib activity with the clinical response was not feasible, because none of the patients for whom organoid cultures were generated were treated with sorafenib (102).

These studies demonstrated the added value tumor organoids may have in the pursuit of precision medicine in treating primary liver cancer. While precision medicine has focused mainly on matching genetic targets with treatments, tumor organoids may be used to validate these matches in in vitro models or discover potential treatment options in the patient, which can be further validated in vivo, using tumor organoid xenograft models.

FUTURE DIRECTIONS OF IN VITRO MODELS OF THE LIVER

Future in vitro models of the liver need to be standardized to satisfy the requirements of (i) high-throughput with ease of use during cell maintenance and (ii) replication of anatomical and metabolic zonation of the liver lobule. Future in vitro models of the liver will combine material advancements made in organ-on-a-chip biotechnologies, bioprinting, and the cell biology advancements in organoid research. This could be achievable using permeable microfluidic tubes lined with LSECs to simulate blood flow and bile excretion or within a modified liver-on-a-chip system, populated by liver organoids and co-cultured with non-parenchymal cells, similar to the early intestinal organoid populated organ-on-a-chip devices

recently developed, which have recapitulated important structural features and functions of the native duodenum (103, 104). Zonal inter-hepatic heterogeneity of the model may be controlled by applying an oxygen gradient across the hepatic cell population. Other considerations include assembly on a matrix that accurately models composition of the in vivo ECM for increased in vivo-like cell–ECM interactions. This would be similar to the bioprinted liver lobules created by Grix and colleagues, where populated HepaRG cells and human stellate cells had microchannel structures, which demonstrated flushing, higher levels of albumin, and CYP450 gene expression, while maintaining overall metabolism (105). The liver organoid-on-a-chip system by Wang and colleagues combined a perfusable organ-on-a-chip system with hiPSC-derived liver organoids, which demonstrated improved cell viability and higher expression of mature hepatic genes and endodermal genes (106).

CONCLUSION

In vitro models of liver disease represent an exciting opportunity to better understand liver homeostasis, response to injury, and cancer development. Conventional methods that use2D primary human hepatocytes and immortalized cell lines or 3D organ explant/PCTS have progressed to using 3D organ-on-a-chip and organoid models with microfluidics and appropriate co-cultures, in which the complex cellular heterogeneity of the originating organ is recapitulated ex vivo. Although well-characterized immortalized cell lines will remain relevant for studying highly conserved cellular processes and interactions, they cannot be regarded as completely accurate models of liver biology in vivo. It is also possible that in the future, as methods become established and validated, in vitro models of the liver will increase the efficacy of pre-clinical drug development, leading to more therapies to treat liver disease. Tumor-derived organoids may also play an essential role in fulfilling the promises of precision medicine, as a method of validating prospective drugs for individual patients.

Conflict of Interest: The authors declare no potential conflict of interest with respect to research, authorship, and/or publication of this manuscript.

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4

Mouse Models of Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma (HCC) represents a major and steadily increasing global health challenge as the most common primary liver malignancy and leading cause of death in cirrhotic patients. The only hope for curative treatment or significant increase in life expectancy is early detection. Once patients have progressed towards end-stage HCC, effective treatment options are extremely limited on the background of a very high degree of heterogeneity in clinical presentation and outcome. Experimental chronic liver injury and cancer have been used extensively to mimic the human disease. In particular, mouse studies have advanced the field due to the ability to easily manipulate the mouse genome and transcriptome for mechanistic evaluations. In addition, they offer the opportunity to screen new therapeutic strategies cost-effectively

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and in quick high-throughput, large-scale formats. The most commonly used mouse models in HCC research can be categorized as chemotoxic, diet-induced, and genetically engineered models. It is important to note that no particular model mimics all features of a given HCC etiology or histological subtype, and each model poses advantages and disadvantages that need to be carefully considered.

Keywords: cirrhosis; hepatocellular carcinoma; hydrodynamic tail vein injection; non-alcoholic fatty liver disease; non-alcoholic steatohepatitis.

INTRODUCTION

Liver cancer is the seventh most common cancer worldwide, and one of the deadliest, with a 5-year survival rate in the range of 5–30% (1). A recent assessment by the Global Burden of Disease Cancer Collaboration revealed that 1 in 38 men and 1 in 111 women will develop liver cancer at some point in their lives (2). In 2016, liver cancer contributed to approximately 10% of all cancer-related deaths worldwide, ranking second in terms of the absolute number of years of life lost, only behind lung cancer (2). High morbidity and mortality rates underscore the need to develop platforms to identify diagnostic tools for better and earlier detection and more effective targeted therapies, in order to halt disease progression and improve survival of patients. Therefore, appropriate preclinical models of liver cancer, in particular hepatocellular carcinoma (HCC)—the most common type of liver cancer, representing approximately 70% of all primary liver malignancies (3, 4)—are critical research tools that enable breakthroughs in the biology of hepatocarcinogenesis and testing of novel therapies. Current therapy options for HCC are limited to surgical and non-surgical ablative therapies or liver transplantation; systemic approaches, such as treatments with multikinase inhibitors, only prolong the life expectancy of patients by 2–3 months (5).

Human hepatocarcinogenesis typically occurs secondary to chronic liver diseases. These include the iron overload disorder hemochromatosis, viral hepatitis, alcoholic fatty liver disease (AFLD), and non-alcoholic fatty liver disease (NAFLD), all of which can promote steatosis, the build-up of excess fat in liver cells, and steatohepatitis, when the condition is accompanied by inflammatory and fibrogenic components. Steatohepatitis causes cellular damage and oxidative stress and instigates the release of pro-inflammatory and pro-fibrogenic signaling molecules, which (i) recruit immune cells to the site of injury, (ii) induce hepatic stellate cell-mediated collagen deposition in the hepatic wound healing response, and (iii) activate liver progenitor cells, as part of a so-called Ductular reaction, to replace lost tissue (6, 7). If injury is halted, matrix is resorbed and normal liver architecture is restored. However, chronic pathological insults can lead to excessive fibrosis, cirrhosis, and ultimately liver cancer (Figure 1) (7).

Micehave become the pillar for biomolecular discovery in human disease due to numerous advantages over other model organisms (8, 9). An ever-growing list of mouse models has been developed to study different aspects of chronic liver

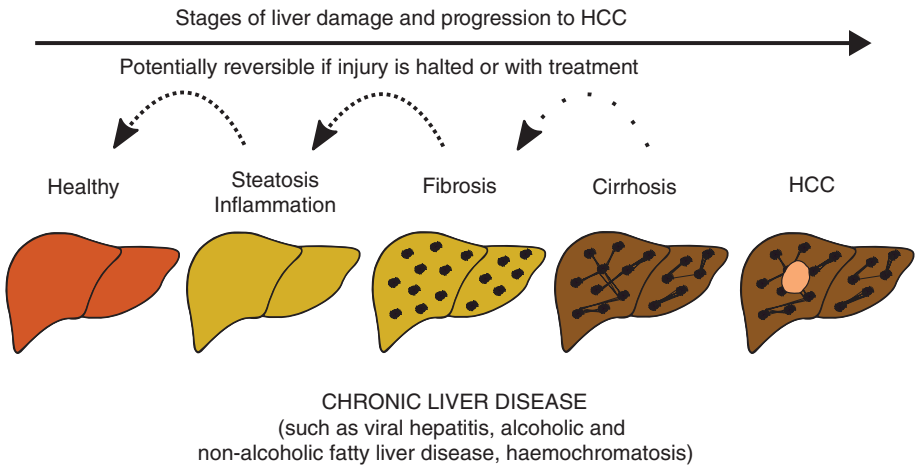

Figure 1 Progressive stages of liver disease to hepatocellular carcinoma development. Chronic liver diseases that predispose to hepatocellular carcinoma (HCC) are generally characterized by steatosis and hepatocellular damage or death, followed by inflammation and fibrosis. These are initial and potentially reversible wound healing when the disease stimulus is withdrawn. However, if the injury is persistent, liver disease may progress to end-stage complications such as cirrhosis and HCC.

disease and progression to HCC. However, appropriate recapitulation of human pathological features has been challenging. Furthermore, when analyzed in detail, distinct models have been shown to induce remarkably different pathological patterns (10). This chapter describes some commonly used as well as the most recently developed mouse models of HCC. Table 1 indicates the timeframe for HCC development and highlights some of the most prominent features and characteristics of each model discussed in this chapter (Table 1).

A variety of strategies can be used to generate HCC in mice. These involve administration of toxic agents, genetic modifications such as expression of oncogenes or disruption of tumor suppressor genes, cancer-promoting diets, and xenograft implantation models. Often, multiple strategies are combined in order to achieve clinically relevant disease progression to mimic human HCC. Thus, it is important to choose the most appropriate model and time point to best answer an underlying research question. Importantly, it may be necessary to validate novel therapeutic targets across multiple models before they can be considered for translation into clinical trials.

CHEMOTOXIC MODELS

Several hepatotoxins have been used to induce HCC in mice. These chemicals either cause DNA damage directly and, therefore, produce cancer-promoting mutations, or act indirectly by facilitating clonal expansion of transformed cells (11). Timing and reproducibility of tumor development can vary significantly between different compounds and, interestingly, between studies from

TABLE 1 **Commonly used mouse models of hepatocellular carcinoma classified as chemotoxic, diet-based, and genetic**

Model	HCC development (time)	Features	References
Chemotoxic			
DEN	Males >80%, females 10–30% incidence at 9 months.	Neutrophil infiltration, bile duct proliferation, centrilobular hemorrhagic necrosis, bridging necrosis. No fibrosis or cirrhosis.	(15–18, 21–27)
CCl_4	1–2 years for most mice strains. As early as 15 weeks in A/J mice	Hepatocyte necrosis, steatosis, Kupffer cell activation, immune cell infiltration. Fibrosis precedes HCC.	(42–52)
TAA	6–12 months	Mild steatosis, centrilobular necrosis, severe inflammation. Steady progressive worsening of inflammatory, fibrogenic and progenitor cell responses. Strong centrally-driven fibrotic component, progressing to cirrhosis prior to HCC.	(10, 58–61, 63–68, 71–74)
DEN+CCl_4	100% incidence at 5 months	Similar to CCl_4 alone but with shortened HCC latency and increased presence of progenitor cells.	(54, 55)
DEN+TAA+HFD	100% incidence at 6 months	Reliable progression to HCC with short latency. Includes steatosis, inflammatory and fibrogenic features.	(75)
Diet			
CDE	75% incidence at 14 months	Periportal injury, severe steatosis at early stages. Mild to moderate fibrosis, strong liver progenitor cell component.	(10, 92, 93)
ALIOS	60% incidence at 1 year	Severe steatosis, hepatic necrosis and inflammation. Ballooning hepatocytes and Mallory hyaline at 16 weeks. Mild periportal to bridging fibrosis, later liver progenitor cell involvement.	(97, 98)
DIAMOND	89% incidence at 1 year (can be accelerated to 6 months by combination with CCl_4).	Pronounced hepatocyte ballooning and progressive fibrosis at 6 months. Strong histologic and transcriptomic similarities with human NASH and HCC.	(99–101)

Table continued on following page

TABLE 1 **Commonly used mouse models of hepatocellular carcinoma classified as chemotoxic, diet-based, and genetic** (Continued)

Model	HCC development (time)	Features	References
MUP-uPA+HFD	78.6% incidence at 32–40 weeks	Several hallmarks of human NASH (activation of hepatic stellate cells, bridging fibrosis, immune cell infiltration, and ballooning hepatocytes), Highlights the role of ER stress in HCC development.	(102, 103)
Genetic			
c-Myc	Incidence of 40% at 45 weeks, 60% at 55 weeks, and 80% at 65 weeks	Mild to severe hepatic dysplasia at 2–3 months of age. First carcinogenic lesions at about 1 year of age. Can be accelerated by co-expression of TGF-α and prevented by HGF. HCC development drastically accelerated by co-treatment with CCl_4.	(109–113)
E2F1	Incidence of 33% at 1 year	Hepatic dysplasia at 2 months and adenomas at 6 months. HCC development accelerated by c-Myc co-expression.	(115, 116)
Active β-catenin +H-ras^{G12V}	100% incidence within 2 months of genetic manipulation	Active β-catenin alone does not progress to HCC. Combination with H-ras^{G12V} induces rapid HCC development. Dysplastic hepatocytes surrounded by immune infiltration in the first 4 weeks and multifocal nodules by 5 weeks.	(122, 123)
Apc KO	67% incidence at 8–9 months	Dose of adenoviral injections is key (0.5×10^9 pfu). High doses increase mortality risk. Trabecular and well-differentiated HCCs.	(124, 125)
Trp53 KO	14–20 months	Majority of tumors display bipotential cell phenotypes (co-expression of hepatocyte and biliary markers).	(127, 129)
PTEN KO	Incidence of 47% at 44 weeks and 66% at 74–78 weeks	Hepatomegaly and steatosis at 10 weeks. Features of NASH (hepatic inflammation and fibrosis) at 40 weeks.	(132, 133)

Active β-catenin, β-catenin gene Ctnnb1 lacking exon 3; ALIOS, American lifestyle-induced obesity syndrome; Apc, adenomatous polyposis coli; CCl_4, carbon tetrachloride; CDE, choline-deficient and ethionine-supplemented diet; c-Myc, Myc proto-oncogene; DEN, diethylnitrosamine; DIAMOND, diet-induced animal model of non-alcoholic fatty liver disease; E2F1, E2F transcription factor 1; HFD, high-fat-diet; H-rasG12V, substitution of glycine with valine at position 12 of human RAS; MUP-uPA, major urinary protein-urokinase-type plasminogen activator transgenic mice; PTEN KO, liver-specific knockout of phosphatase and tensin homolog; TAA, thioacetamide; Trp53 KO, liver-specific knockout of the mouse p53 ortholog.

different research groups. The latter possibly reflects differences in the murine gut microbiota, which are known to play key roles in tumor growth, as recently demonstrated in pancreatic and colon cancer as well as melanoma (12). Hepatotoxins that are generally regarded as appropriate tumor inducers mostly recapitulate the multistep progression stages of human HCC involving injury, steatosis, inflammation, fibrosis, and carcinogenesis. However, the degree and level of interplay of these histological changes often varies. The most commonly used hepatotoxins to study HCC in mice are discussed below.

Diethylnitrosamine (DEN)

DEN, also known as *N*-nitrosodiethylamine, is probably the most commonly used chemical to induce liver cancer in mice and is either administered orally or through peritoneal injection. DEN is bioactivated in centrilobular hepatocytes in a cytochrome P450-dependent manner and produces metabolic sub-products that have DNA alkylating properties, ultimately leading to mutagenesis (13). Tumor incidence is reduced in CYP2 E1-deficient compared to wild-type mice, suggesting that it may be one of the key CYP enzymes that catalyzes DEN bioactivation (13). DEN administration also induces reactive oxygen species (ROS) formation and oxidative stress, which constitutes an additional mechanism by which it promotes hepatocarcinogenesis (14).

A single intraperitoneal dose of 5 mg/kg of DEN to weaning, 15-days-old male mice is sufficient to induce hepatocarcinogenesis in approximately 80% of all animals 9 months after induction (15). However, long-term administration or higher doses of DEN can reach an incidence of 100% in shorter timeframes (15–18). While DEN reliably induces HCC, dose, timing of administration, gender, age, and strain impact the severity and timing of tumor appearance (19). High cellular proliferation is known to enhance mutagenesis by chemical carcinogens both in vitro and in vivo (20). Thus, in most cases, juvenile mice are used, which display actively proliferating hepatocytes at this stage of their development. DEN treatment results in the expansion of cells with oncogenic mutations, leading to dysplastic lesions that eventually give rise to carcinomas (19).

Vesselinovitch and Mihailovich conducted an extensive dose–time response kinetics study of DEN-induced hepatic carcinogenesis, which included evaluation of early alterations such as basophilic foci and nodules, as well as late transformation to adenomas and hepatocellular carcinoma lesions (21). Briefly, HCC developed more reliably when DEN was administrated to younger mice (15-days old) in a dose-dependent manner, ranging from as little as 0.625 up to 5 mg/kg of body weight, whereas treatment of more mature mice (42-days old) with doses up to 50 mg/kg failed to induce predictable carcinogenesis within the same observational period of up to 110 weeks (21).

Another factor influencing the carcinogenicity of DEN is the gender. The incidence for DEN-induced liver cancer can reach 100% for male mice, but is only approximately 10–30% in females, indicating a gender-specific differential response (22–27). Nakatani et al. studied the influence of hormonal factors and demonstrated that ovariectomy or testosterone supplementation increased the occurrence of liver tumors in females treated with DEN. Furthermore, male castration paralleled by estrogen administration resulted in a reduced tumor incidence of 26%, similar to the prevalence observed in females (28).

These findings in experimental liver carcinogenesis are consistent with the observation that men are three to five times more likely to develop HCC than women (29). The precise molecular mechanisms underlying gender imbalance are not completely understood, but have recently been demonstrated to involve estrogen-dependent interleukin (IL)-6 inhibition in females, and direct downstream effects in nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) signaling, two key transcription factors in HCC development (24, 30).

The sequence of hepatic alterations in DEN-treated mice is highly similar to human chronic liver disease to HCC progression. During the course of their life, mice subjected to DEN develop histological alterations that include neutrophil infiltration, bile duct proliferation, centrilobular hemorrhagic necrosis, and bridging necrosis, all of which are observed in human HCC (31). However, the most common histopathological features of human HCC, fibrosis, and cirrhosis (32) are not observed with DEN administration alone (33). Indeed, 80–85% of all cases of HCC occur in cirrhotic patients, and only about 10% of HCC cases are reported in the absence of any chronic liver disease (34). Thus, models that include fibrogenesis are most relevant to a better understanding of the pathogenesis of human disease. The combination of DEN with fibrotic compounds such as carbon tetrachloride (CCl_4) and thioacetamide (TAA) has been demonstrated to better model this particular feature of HCC (35).

Carbon tetrachloride (CCl4)

CCl_4 was widely used as a fumigant, cleaning product, and in fire extinguishers until it was phased out due to safety concerns and banned worldwide in 1996, under the "Montreal Protocol on Substances that Deplete the Ozone Layer." CCl_4 is a hepatotoxin known to induce liver damage, infiltration of inflammatory cells, and fibrosis (36). Similar to DEN, hepatotoxicity involves metabolism through cytochrome P450 and generation of toxic metabolic sub-products in hepatocytes (37, 38). One of them, trichloromethyl radical (CCl_3*), is a highly reactive intermediary that can damage nucleic acids, proteins, and lipids, leading to impairments in diverse cellular processes (39). The main mechanism for CCl_4-induced hepatic toxicity involves exacerbated lipid peroxidation, which leads to plasma membrane damage and secondary accumulation of lipoproteins and lipid droplets in hepatocytes (40, 41). Thus, one advantage of the CCl_4 model is that it includes hepatic steatosis in its pathogenesis. Additionally, CCl_4 promotes activation of Kupffer cells, and this has been demonstrated to be necessary for its fibrogenic effect (42). Pro-inflammatory signaling mediated by Kupffer cells attracts further immune cell recruitment and infiltration, which contributes to the tissue damage elicited by CCl_4 administration (43). A single dose of CCl_4 leads to centrilobular liver necrosis, followed by tissue repair and regeneration (44). Thus, in contrast to DEN, CCl_4 must be administrated chronically and/or repeatedly in order to lead to cycles of injury, inflammation, fibrosis, and cirrhosis, and it eventually gives rise to HCC (11).

CCl_4 is most often provided to mice as a 2–4 mL/kg 50% solution in mineral or vegetal oil, either by gavage or intraperitoneally (45–47). However, a significant variation in dosage can be found in the literature. Inhalation exposure has also been utilized, although this route is much less common (48). The frequency

of doses also varies in different protocols, but generally consists of weekly, biweekly, or three times a week administrations. While the pathology induced by CCl_4 has a prominent fibrogenic component (49, 50) and A/J male mice were shown to present 100% incidence of HCC following a protocol of only 17-weeks of CCl_4 administration (49), the evolution to HCC generally only occurs after long-term exposures of 1–2 years for most mouse strains (11, 51, 52). Therefore, CCl_4 is often combined with other tumor-promoting agents such as alcohol, DEN, and others, which allow for a more timely induction of hepatic carcinogenesis, while maintaining the inflammatory and fibrogenic components that are akin to human HCC (53). For example, a single dose of DEN at 2 weeks of age, followed by biweekly administrations of CCl_4 led to a two fold increase in carcinogenesis at 5 months of age (54, 55). Interestingly, this was associated with significant increase in the expression of progenitor cell markers in the non-cancerous parenchyma, suggesting the role of fibrosis in promoting cellular alterations that lead to carcinogenesis. The presence of cells expressing progenitor features has been associated with a more aggressive tumor phenotype and poorer outcomes in human studies (56, 57). However, the exact role of progenitor cells in the development of HCC is largely unknown and is a subject of intense research in the field (7).

Thioacetamide (TAA)

TAA has mostly been used to induce fibrosis, cirrhosis, and liver cancer, including cholangiocarcinoma in rats, but studies increasingly emerge on hepatic TAA toxicity in mice (10., 58–61). TAA is an organosulfur compound that undergoes a two-step bioactivation through the flavin-adenine dinucleotide-containing monooxygenases or cytochrome P450 via TAA sulfoxide (TASO or sulfine) to thioacetamide sulfdioxide (TASO2 or sulfene). TASO2 is a highly reactive metabolite, which causes significant fat deposition, necrosis, and inflammatory cell aggregates in centrilobular areas, where TAA is metabolized (10, 62). The mechanisms of toxicity are believed to be secondary to its oxidant properties, including lipid peroxidation and production of ROS, dampening antioxidant defenses and exacerbating hepatic oxidative stress (62). In mice, it is usually administered by the addition of drinking water at 300 to 600 mg/L, allowing for a simple model to induce carcinogenesis without the need for regular injections (10, 60, 63). However, it can also be administered intraperitoneally two to five times a week (63–65).

A detailed 6-week time course analysis by Köhn-Gaone and colleagues compared the molecular and cellular injury dynamics of TAA-induced chronic liver injury to feeding of a choline-deficient and ethionine-supplemented (CDE) diet in mice (10). While the CDE diet induced periportal injury, steatosis, and fibrosis with a peak of all measured injury parameters in the first 2 weeks, followed by slow normalization of liver histology and function, TAA supplementation led to progressively worsening inflammatory, fibrogenic, and liver progenitor cell responses. Various studies have reported portal, portal–portal, or portal–central bridging in the TAA model (63, 66–68). However, the comprehensive time course analysis in C57BL/6 mice revealed that fibrosis is centrally driven in TAA liver injury and progresses to cirrhosis within only 6 weeks of treatment (10). Long-term treatment with TAA alone has been

demonstrated to induce HCC within 14–16 weeks in rats (69, 70). Murine studies are much rarer in the literature and describe HCC development after 26 weeks to 12 months of TAA treatment (71–74). Figure 2 illustrates tumor histology and characteristics of TAA-induced HCC development compared to CDE-mediated HCC development in mice after 7 months of treatment.

Often, TAA has been used in combination with other methods to induce hepatocarcinogenesis in a shorter timeframe. For instance, combination of a single dose of DEN at 14 days, accompanied by 300 mg/L of TAA in the drinking water, along with high-fat-diet (HFD) feeding from 4 weeks of age, elicited histological features of inflammation, steatosis, and fibrosis that were significantly exacerbated as early as 12 weeks of treatment, and 100% of animals progressed to liver tumors by 24 weeks (75). Such combinatory strategies not only recapitulate different features of human HCC but may also significantly reduce the time for tumor development and have therefore become quite popular in liver cancer research (76).

DIET-INDUCED MODELS

Recent advances in antiviral therapies, which can result in permanent suppression of hepatitis B virus (HBV) and eradication of HCV, are significantly reducing the incidence of HCC from viral etiology (77). Furthermore, the second and most prevalent risk factor for HCC, alcoholic steatosis, is relatively stable over time (78). This can be attributed to observations pointing to steady or decreasing trend of alcohol consumption per capita in most countries over the past decades (79). Therefore, NAFLD and its advanced form, non-alcoholic steatohepatitis (NASH), are responsible for the current and future increases in chronic liver disease and HCC incidence worldwide (80). NAFLD is the hepatic manifestation of the metabolic syndrome and is estimated to affect an astonishing 24% of the world population (81). NAFLD is generally accompanied by obesity, insulin resistance, and dyslipidemia. Thus, diet-induced models that manifest those metabolic alterations are well suited to represent human NAFLD-driven HCC. Noteworthy, only a small fraction of patients with NAFLD progresses to HCC (82). The network of factors that predict NAFLD progression to NASH and carcinogenesis are poorly understood, hence the importance of appropriate pre-clinical models to further our knowledge in this particular setting (83, 84).

Several diet-induced mouse models exist that induce HCC; however, not all models replicate all the associated metabolic dysfunctions that are characteristic of human NAFLD. Feeding rodents a diet deficient in choline (CD), for instance, is known to induce hepatic steatosis and progression to HCC (85). This has first been observed in rats (86, 87) and confirmed in a number of mouse strains (88). Mechanistically, choline deficiency leads to defects in phospholipid synthesis, lipoprotein secretion, oxidative damage, endoplasmic reticulum (ER) stress, and cell death (85). Diets that combine choline with methionine deficiency (MCD) induce even more severe pathology, characterized by steatohepatitis within 1–2 weeks and fibrosis by 8–10 weeks of feeding (89, 90). However, both CD and MCD are not accompanied by other physiological hallmarks of the metabolic syndrome, namely dyslipidemia, glucose intolerance, and insulin resistance (91). Furthermore, these diets promote severe body weight loss and morbidity,

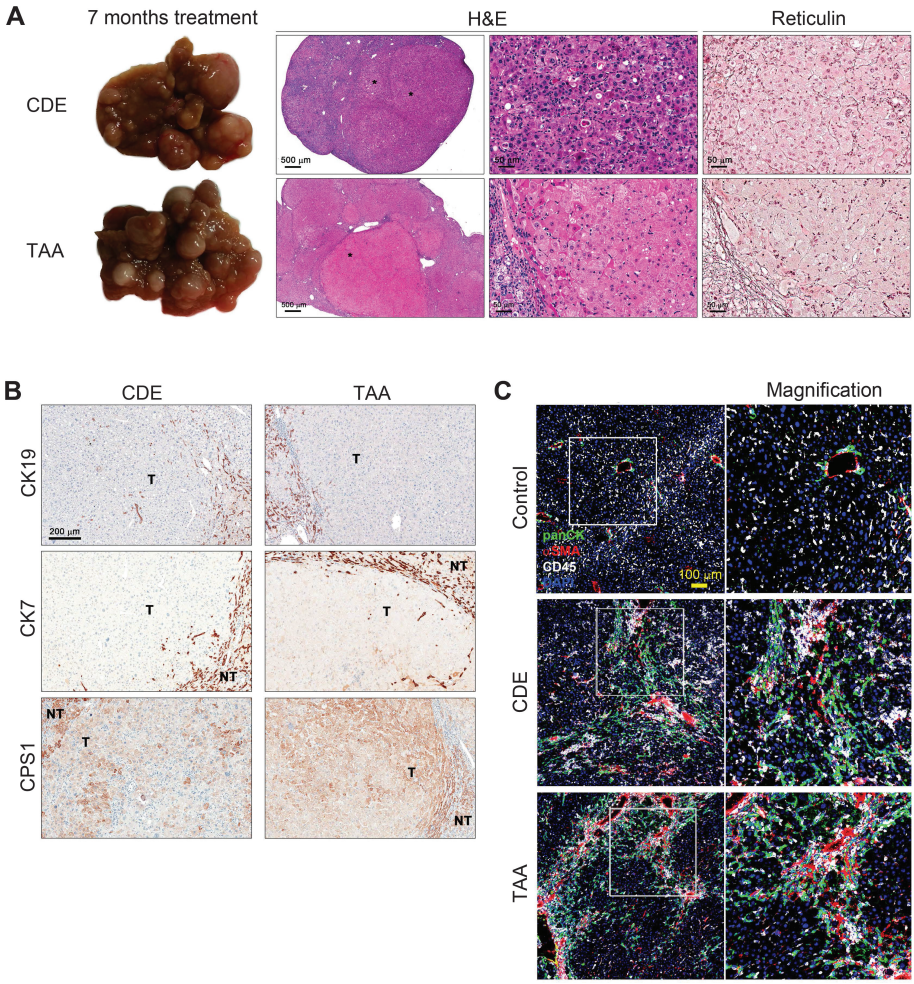

Figure 2 Histology and cellular characterization of CDE- and TAA-induced tumors. (A) Gross liver morphology assessment of C57BL/6J mice treated with CDE or TAA for 7 months demonstrates the development of advanced tumors. Hematoxylin and eosin (H&E) stains reveal the presence of a great variety of neoplastic changes and nodules. TAA-induced tumors comprise large polygonal cells with abundant eosinophilic cytoplasm and atypical stripped nuclei as well as moderate basophilic cell infiltrates. Similar characteristics are identified in CDE-induced tumors, although with weaker eosinophilic cytoplasm and enriched small basophilic cells. An altered stromal network of collagen III-composing fibers demonstrates the characteristic thickening of hepatic cell plates and diffuse reticulin structures within tumors. Reticulin crowding at the interface of tumorous and non-tumorous tissue indicates invasive tumor growth into the surrounding tissue. (B) Immunohistochemical staining of both CDE- and TAA-induced tumors with the biliary and liver progenitor cell markers cytokeratin 19 (CK19) and CK7 and the hepatocellular carcinoma (HCC) marker carbamoyl phosphate synthetase I (CPS1) identifies the tumors as HCC with biliary and progenitor proliferation almost exclusively in extra-tumoral tissue. (C) Fluorescent labeling of the cellular components of the injury and regeneration niche, which hosts panCK+ biliary and progenitor cells (green), alpha-smooth muscle actin (aSMA)+ hepatic stellate cells (red) and CD45+ inflammatory cells (white), illustrates the cells' close spatial relationship and potential for cellular cross-talk in the tumor-surrounding tissue of CDE and TAA mice. NT, non-tumor; T, tumor.

and thus, they are generally not suitable for long-term experiments leading up to HCC. In order to significantly reduce the time to carcinogenesis, ethionine, a non-proteinogenic cytotoxic methionine analogue, can be added to the CD diet (CDE) and induce severe hepatic inflammation with a strong proliferation of liver progenitor cells and activation of fibrosis-driving hepatic stellate cells in as early as 2 weeks of feeding (92). Long-term CDE feeding of C57Bl/6 mice induced HCC in 75% of animals, whereby tumor development was significantly inhibited following treatment with the multi-tyrosine kinase inhibitor imatinib mesylate (93). Features of CDE-induced HCC after 7 months of treatment are demonstrated in Figure 2 in comparison with tumors provoked by TAA administration.

The HFD model involves feeding animals *ad libitum* diets containing a total of 45% to75% calories intake derived from fats (94). Several formulations exist in which the types and percentages of fats vary significantly (95). In this model, hepatic steatosis, characterized by increased liver triglyceride accumulation and fatty acid synthesis, is accompanied by other features of the metabolic syndrome such as obesity, glucose intolerance, and insulin resistance (96). It is a very reliable model to induce simple hepatic steatosis; however, in most mouse strains, no additional liver damage and inflammation, neither development to HCC, are observed (83).

To produce a model that more closely resembles the human disease, Tetri et al. developed a formulation containing nutrients commonly found in fast foods and kept mice under conditions designed to encourage sedentary behavior, the so-called American Lifestyle-Induced Obesity Syndrome (ALIOS) model (97). The formulation included trans-fats and high-fructose corn syrup, in addition to removal of cage racks to promote low energy expenditure. After 16 weeks under this regime, mice developed severe hepatic steatosis, associated with necrosis and inflammation. Histological features of human NASH such as ballooning hepatocytes and Mallory hyaline were also described. Fibrogenesis was not detected histologically; however, procollagen mRNA expression was found to be upregulated, suggesting that fibrosis might develop at time points later than 16 weeks (97). Consistent with this hypothesis, a separate study used 12-month exposure to the ALIOS protocol and revealed fibrosis with severity ranging from mild periportal to bridging fibrosis (98). These observations were accompanied by substantial activation of the liver progenitor cell niche, which was evidenced through increased numbers of cells positive for pan-cytokeratin (panCK) and sex-determining region Y-box 9 (Sox9) throughout the parenchyma. HCC was observed in 60% of all ALIOS mice at 12 months (98). These studies demonstrate that diet and lifestyle interventions are sufficient for the induction of NASH and hepatocarcinogenesis in mice. Future studies are necessary to assess the level of similarities between the genetics and transcriptomics of carcinogenesis observed in ALIOS mice compared to human HCC.

Another western diet model, comprised of high cholesterol, high saturated fat, and high fructose, has been shown to promote NASH, which was characterized by pronounced hepatocyte ballooning and progressive fibrosis after 6 months of feeding (99). No HCC was observed at 6 months, but it resulted in 89% incidence of spontaneous HCC after 12 months of feeding to a stable isogenic cross between C57BL/6J and S129 mice (100). Interestingly, this model, named Diet-Induced

Animal Model Of Non-alcoholic fatty liver Disease (DIAMOND), presented remarkable histologic and transcriptomic similarities with human NASH and HCC. Tsuchida et al. recently demonstrated that the same diet, combined with low weekly doses of CCl_4, can develop rapid progression to stage 3 fibrosis and HCC within 12 and 24 weeks, respectively. The pathology closely mimicked histological, immunological, and transcriptomic features of human NASH, thus representing a rapid induction model suitable to study hepatocarcinogenesis in a clinically relevant setting (101).

Most recently, another diet-induced model of NASH-driven HCC was established through genetically induced predisposition to HCC. Feeding of a HFD to major urinary protein (MUP)-urokinase-type plasminogen activator (uPA) transgenic mice, which overexpress uPA specifically in hepatocytes, induced liver disease that recapitulated several hallmarks of human NASH and reliable progression to HCC (102). In comparison with HFD-fed control wild-type animals, MUP-uPA mice displayed increased activation of hepatic stellate cells as well as upregulation of collagen and other fibrogenic markers. Immune cell infiltration, bridging fibrosis, and ballooning hepatocytes were all present in MUP-uPA mice at 24 weeks after diet initiation. Hepatocarcinogenesis was observed at 32–40 weeks of HFD feeding in about 78.6% of these animals. The mechanism of disease progression involved excessive ER stress, induced by hepatocyte overexpression of uPA and exacerbated by HFD, as well as tumor necrosis factor (TNF)-dependent inflammation (102). This model was later used to demonstrate the key role of caspase-2, downstream of TNF and ER stress, in mediating the activation of sterol regulatory element-binding proteins (SREBP), recognized to participate in NASH development (103).

GENETICALLY ENGINEERED MODELS

Human HCC is known to have a very high level of inter- and intra-tumor genetic heterogeneity (104). Illustrating this concept, a study by Schulze and colleagues employed exome sequencing analysis and identified mutations in 161 distinct putative driver genes in a cohort of only 243 hepatic tumors (105). In addition, a recent study published by the Cancer Genome Atlas Research Network investigated 383 HCC cases by whole-exome sequencing and DNA copy number analyses and assessed 196 HCC samples for their DNA methylation, RNA, miRNA, and proteomic status. The comprehensive data set, coupled with robust statistical power by the large investigated cohort, enabled the identification of various molecular signatures, which may be therapeutically targeted in different HCC subgroups (106). The individual roles of many of the identified genes and pathways in hepatocarcinogenesis have been, and continue to be, determined using mouse genetics. Such models are excellent tools to investigate the discrete effects of candidate oncogenes or tumor suppressor genes in HCC. The number of genetically engineered mouse models of HCC is very large and continuously expanding. Moreover, comprehensive reviews describing available models have been published elsewhere (107). The scope of this chapter is to present a few of the most commonly used and well-characterized genetic models in detail.

c-Myc

The transcription factor c-Myc controls numerous cellular processes, including cell cycle progression and proliferation. Mutations that activate c-Myc are known to be highly associated with carcinogenesis in human HCC (108). Transgenic mice, overexpressing c-Myc specifically in the liver, develop hepatic tumors with a relatively long latency of approximately 12–15 months (109–111). Tumor incidence is of about 40% at 45 weeks of age, 60% at 55 weeks, and 80% at 65 weeks (111). To limit oncogene expression to the hepatic tissue, the albumin promoter has most commonly been utilized due to its specificity to hepatocytes (11). When c-Myc was combined with transgene expression of growth factors such as transforming growth factor alpha (TGF-α) and hepatocyte growth factor (HGF), opposing results were obtained. Combination with TGF-α led to a significant acceleration of the neoplastic development, with tumors developing before 16 weeks of age (110). In contrast, HGF prevented malignant transformation when investigated in a similar setting (112). Interestingly, the kinetics of carcinogenesis induced by c-Myc can be drastically accelerated to under 40 days by co-treatment with the hepatotoxins CCl_4 or 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (113). An elegant study by Shachaf and colleagues demonstrated that inactivation of the MYC oncogene resulted in tumor regression, accompanied by differentiation of the tumor cells into hepatocytes and cholangiocytes. The tumors lay dormant until c-Myc was reactivated in cells the authors proposed to be cancer stem cell-like cells (114).

The E2F transcription factor 1 (E2F1) has also been identified as frequently dysregulated and/or mutated and was demonstrated to induce HCC in mice upon hepatic transgenic overexpression (115, 116). E2F1 mice showed signs of hepatic alterations as early as 2 months of age, with most animals developing adenomas at 6 months. However, tumors progressed to HCC only in one-third of experimental animals at 1 year of age. Co-expression of E2F1 and c-Myc further accelerated the appearance of focal lesions and severe dysplasia, leading to earlier development of HCC as compared to either of the oncogenes alone (117).

Wnt/β-catenin

Wnt/β-catenin signaling controls a plethora of cellular communication networks in embryonic development and demonstrates key roles in regulating stemness and cell differentiation in health and disease (118). Pathological alterations of this pathway are known for its involvement in many human cancers, including liver cancer (118, 119). Beta-catenin is one of the key effectors of Wnt signaling, and its gene *CTNNB1* is the most frequently found mutated component of this pathway in human HCC (120). Altered activity of β-catenin, as evidenced by mutation or nuclear translocation, was observed as an early event in hepatocarcinogenesis driven by distinct genetic models (121). Yet, constitutive activation of β-catenin through deletion of its regulatory domain was not sufficient to promote hepatic tumorigenesis by itself (122). When combined with oncogenic H-ras (H-ras^{G12V}), however, β-catenin activation resulted in aggressive HCC development with 100% incidence within 3–4 months following the genetic manipulation (123).

Interestingly, the most commonly used approach to induce hepatic β-catenin activation in mice has been the selective Cre-Lox disruption of its negative regulator, adenomatous polyposis coli (Apc). This indirect approach for the stabilization of active β-catenin was shown to lead to spontaneous hepatocyte hyperplasia and 67% incidence of HCC in the surviving animals following 8–9 months of model establishment (124). Tumors generated through this model have recently been shown to possess a unique metabolic signature, characterized by exacerbated fatty acid utilization (125), suggesting that inhibition of fatty acid oxidation could be a potential therapeutic approach for β-catenin-induced HCC.

Liver-specific knockout models

Liver-specific knockout models of tumor suppressor genes have also been developed and utilized either alone or in combination with other insults to study liver cancer. The *TP53* gene that encodes for the tumor suppressor p53 is found mutated in most human cancers, including HCC (126). P53 knockout mice displayed a significantly increased hepatocyte proliferation rate and LPC-like cells in periportal liver regions (127). In addition, p53-/-LPCs isolated from these mice and injected subcutaneously into athymic nude mice generated tumors with a HCC morphology (128). In a separate study, the homozygous deletion of *Trp53* (the mouse ortholog), specifically in the liver, led to HCC formation after 14 months of age (129). The authors also showed that p53 deletion gave raise to tumors with a bilineal phenotype and increased proliferation of liver progenitor cells. These observations support a model in which loss of function of p53 may promote HCC through an increase in the proliferative capacity of progenitor cells.

Another ubiquitously expressed tumor suppressor gene frequently found mutated or downregulated in human HCC is phosphatase and tensin homolog (PTEN) (130, 131). Its phosphatase activity inhibitsphosphatidylinositol-3-kinases (PI3K) and consequently suppresses downstream protein kinase B (PKB/Akt) and mammalian target of rapamycin (mTOR) growth-promoting signaling. Horie et al. generated a liver-specific PTEN knockout model through the crossing of albumin promoter Cre mice with PTEN-floxed mice (132). The resulting conditional PTEN knockout mice showed hepatomegaly and steatosis as early as 10 weeks of age. At 40 weeks, features of NASH including hepatic inflammation and fibrosis were observed. This model also reliably progressed to tumorigenesis, although with a long latency. The incidence of hepatic adenomas was 47% at 44 weeks, and by 74–78 weeks, 66% of animals displayed HCC (132). The mechanism of carcinogenesis driven by PTEN disruption has later been addressed. It involves hepatic injury-dependent expansion of epithelial cellular adhesion molecule (EpCAM), alpha-fetoprotein (AFP), and cytokeratin 19 (CK-19) positive progenitor cells (133).

Hydrodynamic tail vein injection (HTVI) models

Traditionally, genetic modifications, such as gene knockouts, knockins, and transgene overexpression, were introduced in the germline of parent mouse strains to produce whole body genetic alterations (134). This approach has been extensively used in HCC research; however, advances in genetic manipulation tools, such as

Cre-Lox recombination, have allowed for a much more precise spatial and temporal control of candidate genes expression, and their utilization has become widespread (135). Notably, this kind of approach has been used to produce conditional liver-specific genetic manipulations, overcoming the problem of embryonic lethal gene mutations as well as restricting phenotypes to the hepatic tissue. Nonetheless, these are both expensive and time-consuming strategies.

A simple and inexpensive alternative method to transfect and gene-edit hepatocytes in vivo is represented by hydrodynamic tail vein injection (HTVI) of "naked" plasmid DNA (non-viral vector, not associated with protective proteins or lipids) directly into the liver of adult mice (136, 137). The technique consists of rapid injection of a large amount of plasmid DNA (about 50 μg in a volume of saline that signifies 10% of the body weight of the injected mouse) into the mouse lateral tail vein (138). HTVI results in transient heart dysfunction and fluid accumulation in the inferior vena cava. The enormous hydrodynamic pressure then forces the fluid into the liver in a retrograde movement, enlarges the liver, and pushes the plasmid DNA into hepatocytes via enlarged sinusoidal fenestrae and transient membrane pores (139), with transfection efficiencies ranging from around 10 to 40% of all hepatocytes (136, 140). While the liver is primarily targeted, other organs including heart, kidney, lung, and spleen are also affected by HTVI; however, to only 0.1% of the levels achieved in the liver (138, 141). One caveat of HTVI is that the expression of transfected genes is transient, peaking within 24 h, but dropping dramatically thereafter (142). In order to circumvent this pitfall, HTVI has been combined with DNA recombination technologies such as Cre-Lox, sleeping beauty (SB) transposase, and CRISPR-Cas9, allowing for genomic integration and continuous expression of genetic modifications. HTVI has been used increasingly to study genetic factors influencing HCC biology, and a variety of HCC models have recently been developed using this approach. Particularly, HTVI permits the assessment of more than one genetic alteration at the same time and therefore the investigation of the combinatory effects of targeting multiple pathways simultaneously. A number of models created through HTVI are discussed below and are summarized in Figure 3.

The PI3K/AKT/mTOR pathway is central in the regulation of hepatocyte cell metabolism, growth, and proliferation (143). Upregulation of this pathway is frequently observed in human HCC (144, 145) and is associated with poorer outcomes (146). As described before, conditional liver knockout of PTEN, a negative regulator of this pathway, induces NASH-like liver disease and HCC in mice (132). HTVI was used to introduce a constitutively active form of AKT (myristoylated AKT) that induced HCC about 6 months post-injection (147). Wild-type mice were co-injected with SB transposase and active AKT vectors for somatic integration. Disease progression resembled that of PTEN knockout mice, with increased lipogenesis and upregulation of genes involved in fatty acid synthesis (147). Importantly, the expression of transfected AKT was observed in relatively few cells, which were surrounded by non-transfected hepatocytes. This is considered to be an advantage of HTVI, in that it better resembles human liver cancer, where only a limited number of foci are believed to give rise to HCC (140).

It was later reported that co-injection of AKT along with H-ras^{G12V} significantly increases the kinetics of liver tumor development, with 100% of injected animals presenting tumors within 4 weeks post-injection (148). Similar kinetics were observed when AKT was co-expressed with active β-catenin (149).

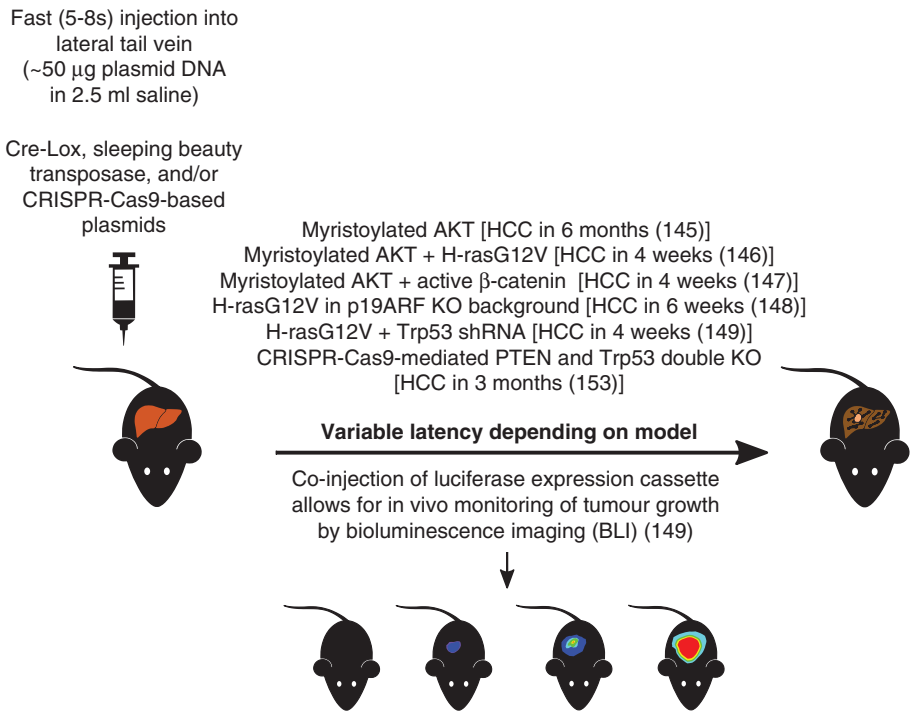

Figure 3 Hydrodynamic tail vein injection models of hepatocellular carcinoma. Plasmid DNA containing Cre-Lox, sleeping beauty transposase, and/or CRISPR-Cas9-based recombination sequences along with key oncogenes or tumor suppressor disruption sequences is injected into the lateral tail vein of young mice. HCC develops with variable latency depending on the chosen genetic manipulation, as indicated. Co-injection of luciferase expression cassette allows for in vivo monitoring of tumor growth by bioluminescence imaging (BLI). HCC, hepatocellular carcinoma; KO, knockout.

Interestingly, injection of H-ras^{G12V} alone did not lead to HCC (148, 150). However, when expressed in p19 Arf knockout mice, H-ras^{G12V}induced HCC in all mice within 6 weeks that otherwise did not develop with simple disruption of this positive regulator of p53 (150). In a similar way, H-ras^{G12V} combined with a shRNA construct targeting p53 led to hepatocarcinogenesis within only 4 weeks (151). This study also demonstrated that oncogenic vectors can be combined with a SB transposase plasmid containing a firefly luciferase expression cassette, allowing for tumor growth to be monitored non-invasively over time through bioluminescence imaging. Altogether, these results highlight the suitability of HTVI to create models that allow for the utilization of synergistic pathways in promoting HCC for rapid induction studies and proof-of-concept therapeutic interventions.

Notably, the SB transposase system permits efficient genomic recombination of exogenous sequences; however, this approach is not specific in terms of the integration site (152). Recombination can potentially occur virtually anywhere in the

genome, causing undesired disruption of off-target genes (153). The CRISPR-Cas9 system, on the other hand, offers sequence-specific direct editing of DNA with only rare off-target mutations (154). In a proof-of-concept study, Xue et al. utilized HTVI to generate CRISPR-Cas9-mediated knockouts of PTEN and p53, or combinations of the two, that phenocopied the traditional conditional liver knockouts of these genes (155). PTEN and/or p53 disruptions were shown to be present in 3 to 6% of all hepatocytes. At 3 months post-injection, all animals with double disruptions developed liver tumors (155). The same approach was used to target β-catenin and simultaneously introduced a constitutively active version of it. This notoriously less-efficient event, observed in only 0.5% of all hepatocytes, however, demonstrated that CRISPR-Cas9 can also be used to introduce gain-of-function mutations, providing a highly specific and low off target method for the evaluation of novel gene roles in HCC development (155). Consequently, CRISPR-Cas9 combined with HTVI has been increasingly utilized for HCC research in recent years (156–158).

CONCLUSION

HCC poses a global health challenge in terms of prevention, diagnosis, and treatment. While epidemiological studies can provide information on associations between HCC and variables that influence its natural history, the progression of interventions into clinical practice requires demonstrated benefits and safety from carefully designed in vivo models such as the mouse models described here. A large variety of experimental chronic liver injury and HCC models are currently available, each with particular characteristic features, mimicking diverse etiologies and disease progression patterns. Selection of the most appropriate animal model allows for the study of disease context-specific as well as common carcinogenic mechanisms and screening of novel therapeutic targets for clinical translation. Until the tyrosine kinase inhibitor sorafenib was approved for unresectable HCC in 2007, there was no FDA-approved therapies available for patients in advanced-stage chronic liver disease. The improved understanding of the molecular mechanisms of HCC, primarily obtained from several of the animal models herein described, has culminated in the development of current targeted therapies, including sorafenib and regorafenib. Several other agents have been tested, or are currently under clinical evaluation, and will hopefully contribute to improved HCC outcomes in the years to come. There is no perfect animal model for human disease; however, mouse models are still invaluable and will continue to form the cornerstone of preclinical studies, designed to delineate interventions that are effective and safe in the future.

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5

The Role of Lipids in Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma is the fastest growing cause of cancer-related mortality worldwide, with few treatment options and a 70% recurrence rate. This trend is driven largely by the recent surge in incidence of metabolic syndrome, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis. Given the central role of the liver in lipid homeostasis, altered hepatic lipid metabolism has been identified as a contributing factor to hepatocellular carcinoma. Neoplastic cells are highly dependent on lipid metabolism as a source of energy and to support rapid cell division, and fatty acid derivatives play key roles in cell signaling. Aberrant expression of liver fatty acid–binding protein and changes in the ratio of saturated to unsaturated triacylglycerols have been shown to be associated with disease severity and subtype. This chapter focuses on metabolic reprogramming and dysregulation of lipid metabolism as hallmarks of hepatocellular carcinoma.

Keywords: carcinogenesis; fatty acid metabolism; lipidomics; lipid metabolic reprogramming; non-alcoholic steatohepatitis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fastest growing cause of cancer-related mortality, with 840,000 new cases per year worldwide, nearly half of which are in China alone, due in part to the high (5.4%) incidence of chronic hepatitis B virus (HBV) infection (1–3). The incidence of HCC in the United States has tripled over the last four decades, resulting in more than 30,000 new cases and 20,000 deaths per year (1, 4, 5). HCC accounts for 75–85% of cases of primary liver cancer and is the sixth most common cause of cancer and the fourth most common cause of cancer-related death (3). A number of approaches have been developed to treat HCC, including liver resection, ablation, and transplantation. However, the disease is often asymptomatic at early stages and defies early detection. While the short-term prognosis for HCC has improved, the long-term prognosis remains poor, with a 5-year survival rate of 17% (6–8). Surgical resection provides the best chance for recovery, but the cancer is often detected too late for the treatment to be effective, and only about 15% of patients are eligible (9). Even in the case of successful resection, the 5-year rate of HCC recurrence remains about 70 % (10).

HCC is a complex disease with a number of known or suspected etiologies, including hepatitis B or C virus infection; non-alcoholic steatohepatitis (NASH); hemochromatosis; alcohol abuse; primary biliary cirrhosis; α-1 antitrypsin deficiency; Wilson's disease; and carcinogens such as aflatoxin B_1, thorotrast, polyvinyl chloride, and carbon tetrachloride (11, 12). However, inflammation associated with viral hepatitis and fatty liver disease is thought to be a common cross-etiological factor that drives the development of over 90% of liver tumors (13). HBV accounts for 85% of HCC cases in endemic regions such as Southeast Asia and sub-Saharan Africa, whereas HCV is the leading risk factor for HCC in Europe and North America. While HBV and HCV have traditionally driven the majority of HCC cases, the proportion of non-viral HCC cases, especially due to NASH, is expected to increase exponentially, and the overall number of HCC cases is expected to skyrocket over the next decade due to increasing incidence of obesity and diabetes (14). Estes et al. projected that by 2030, the number of NAFLD cases in the United States will increase by 21%, NASH cases by 63%, and HCC cases by 137% (14).

The incidence of fatty liver-associated HCC is increasing in many western countries due to the alarming increase in the number of adults and children with obesity, diabetes, and metabolic syndrome (15). Lipid metabolism is among the liver's most critical functions. Along with proteins, carbohydrates, and nucleic acids, lipids represent one of the four main classes of biomolecules. Starvation depletes fat reserves and causes muscle wasting, whereas excessive caloric intake accompanied by lack of physical activity can lead to obesity, in which fat accumulates in the liver and adipose tissue, disrupting lipid homeostasis and promoting insulin resistance. Altered lipid metabolism is thought to induce inflammation and promote fibrosis (16). Defined as having a body mass index (BMI) greater than 30 kg/m^2, obesity is one of the greatest public health challenges of this century, affecting 700 million people worldwide (17). Now classified as a disease in its own right, obesity is a leading preventable cause of death and is associated with increased risk of diabetes, cardiovascular disease, depression, and several types of cancer, including esophageal adenocarcinoma, leukemia, non-Hodgkin's

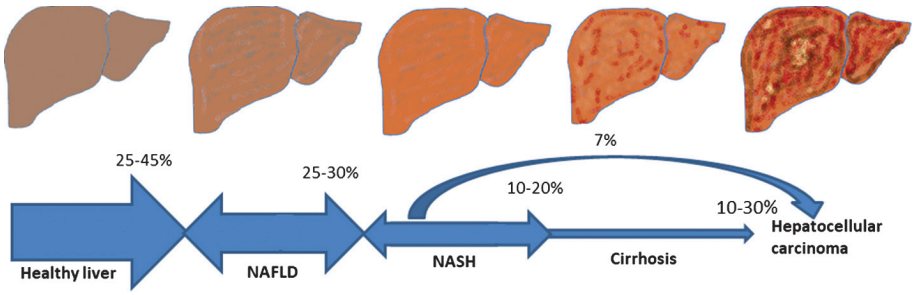

Figure 1 Potential routes of progression from fatty liver to hepatocellular carcinoma. Most steps are at least partially reversible with lifestyle changes, but a fraction of patients at each stage progresses to more severe liver inflammation and fibrosis until loss of hepatic function becomes mostly permanent. In some patients, HCC can develop on a more rapid course without cirrhosis.

lymphoma, multiple myeloma, malignant melanoma, and endometrial, colorectal, breast, prostate, thyroid, and renal cancers (18). Lipids play an intriguing role in the development of HCC, and factors associated with lipid and energy dysregulation, such as obesity (19), diabetes (20), and hepatic steatosis, are risk factors for HCC (21). Although the different viral, genetic, chemical, and metabolic etiologies of HCC vary with respect to early events leading to hepatocarcinogenesis, they increasingly converge on a set of shared biochemical pathways, of which lipid metabolism is a central player. For example, HBV infection, alcoholic liver disease, and NASH lead to increased lipogenesis and reduction of lysophosphatidylcholine (22, 23).

The pathophysiology of metabolic syndrome is complex and involves multiple organ systems, but in the liver the presence of excess fat promotes inflammation and can lead to cycles of liver cell injury and repair. Damage to the liver is often progressive and can result in fibrosis and eventually cirrhosis, but the process is often partially reversible at the early stages with changes in diet and lifestyle (Figure 1).

NAFLD, CIRRHOSIS, AND HCC

While the long-term progression from NAFLD to NASH to cirrhosis to HCC over a period of decades is frequently observed, there are exceptions. Being overweight increases the risk of HCC by 17%, and obesity increases the risk by 89% (24). In patients with chronic HCV or HBV infection, the presence of NAFLD has been shown to increase the risk of oncogenesis in a synergistic manner (25), suggesting that lipid dysregulation is an independent risk factor for HCC.

NAFLD is on track to become the leading cause of non-cirrhotic HCC, in which liver cancer develops independently of cirrhosis (26–29) and might contribute to cryptogenic cirrhosis, in which the otherwise non-symptomatic cirrhosis is discovered incidentally (30, 31). The mechanism underlying cryptogenic HCC is unclear but may involve progression from NAFLD-based steatosis to lipid catabolism such that the underlying steatosis is no longer observable (32, 33).

Some aspects of lipid dysregulation, such as attenuated lysophosphatidylcholine levels, have been found to be common in patients with NASH and cirrhosis (34), whereas levels of the non-essential amino acids such as valine and isoleucine were elevated in patients with cirrhosis (35, 36). Aside from its direct risks, cirrhosis is also the primary risk factor for HCC, and more than 90% of patients with HCC have cirrhosis (37). In a study of 34,932 patients with cirrhosis, 1,960 patients developed HCC (5.6%) within 1.3 years (37). Currently, NASH-related cirrhosis accounts for about 10% of liver transplantations (38).

HBV AND HCC

Several studies have reported changes in lipid metabolism associated with HBV infection and liver regeneration. Park et al. reported significant changes in phosphatidylcholine composition in HBV-infected mouse livers and found that expression of choline-phosphate cytidylyltransferase A (PCYT1A) was significantly delayed (39). Using an HBx transgenic mouse model, Teng et al. tracked changes in lipid profiles during HBV-induced HCC and observed a biphasic peak in triglyceride, cholesterol, and fatty acid levels in serum and liver tissue (40). The first peak was associated with non-specific pro-inflammatory responses to oxidative stress in mouse hepatocytes. Lipid profiles then transiently resolved at 6 months before peaking again at 12 months, representing a terminal metabolic shift and formation of fatty nodules. The peaks were associated with the upregulation of the following five lipid metabolism-related genes, which were subsequently validated in human HBV-related HCC tumors: arachidonate 5-lipoxygenase, lipoprotein lipase, fatty acid–binding protein (FABP) 4, 1-acylglycerol-3-phosphate O-acyltransferase 9, and apolipoprotein A-IV (40). These results suggest that HBV-mediated perturbation of lipid metabolism plays a role in the mechanism of hepatocarcinogenesis.

DE NOVO LIPOGENESIS IN HCC

Given the role of lipids as structural, signaling, and energy storage molecules, there are a number of ways that lipid dysregulation could contribute to hepatocarcinogenesis. Increased ***de novo*** lipogenesis and enforced glycolysis appear to be hallmarks of liver cancer (41). One reason for this is the severe metabolic stress experienced within the poorly vascularized tumor core during rapid proliferation causing a nutrient-poor, hypoxic microenvironment on the brink of necrosis or apoptosis. Fatty acid oxidation can continue to provide energy required for cellular metabolism after glycogen has been depleted and in the absence of glucose from the blood. Therefore, glycolysis is elevated, and lipid catabolism is strongly and characteristically upregulated in HCC and other cancers as a means of cell survival. In rapidly proliferating tumor cells, elevated lipid catabolism helps to satisfy the high energy demands of the cells via acetyl-CoA, NADH, and FADH and supplies glycerophospholipids for cell membrane formation (42).

LIPID METABOLIC REPROGRAMMING

Although not fully understood and not as well studied as changes in glucose or glutamine metabolism, lipid metabolic reprogramming appears to be an effective strategy to sustain cancer stem cells in a hypoxic environment (43, 44). Tumor cells harboring beneficial metabolic changes, including those that produce pro-oncogenic metabolic intermediates, may undergo clonal selection (45). For example, the Warburg effect is a well-known adaptive strategy in which tumor cells forsake normal oxidative phosphorylation in exchange for less-efficient aerobic glycolysis, even in the presence of oxygen (46). Similarly, glutaminolysis helps to sustain the Krebs cycle via increased production of citrate and α-ketoglutarate through elevated glutamine metabolism (47). Changes in fatty acid metabolism through metabolic reprogramming have also been found to play an important role in facilitating carcinogenesis (48). Generally, cells import fatty acids and other lipids from the blood, but HCC tumors upregulate genes involved in fatty acid biosynthesis, including SREBP-1-regulated genes such as ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), FAS, SCD-1, and GPAT, in order to generate fatty acids *de novo* (48, 49). A number of key enzymes, including ACLY, ACC, and fatty acid synthase (FASN), catalyze biosynthesis of fatty acids from citrate and acetyl-CoA. ACC catalyzes a key initial step in fatty acid biosynthesis, the conversion of acetyl-CoA to malonyl-CoA (50), and helps to sustain HCC tumors experiencing metabolic stress (51). As a rate-limiting enzyme in lipogenesis, elevated expression of ACCα has been reported to be an independent predictor associated with poor HCC prognosis (51). Similarly, FASN is a multifunctional enzyme that catalyzes the synthesis of long-chain saturated fatty acids during one of the final stages of fatty acid biosynthesis. FASN is over expressed in HCC as well as in many other types of cancer (52), and genetic ablation and drug targeting studies of FASN have revealed suppressed development of HCC (41, 53, 54). Interestingly, aspirin has been shown to suppress abnormal lipid metabolism in hepatoma cells via NF-κB targeting by downregulating the expression of acyl-CoA synthetase long-chain family member 1 (ACSL1), which converts free fatty acids into fatty acyl-CoA esters, an early step in fatty acid degradation and important for lipid biosynthesis (55).

To exploit fatty acids as an efficient source of stored energy, β-oxidation has also been shown to be upregulated in HCC and other cancers (49, 56, 57). In particular, 2-oxoglutarate is upregulated in HCC, and the levels of pyruvate and lactic acid are elevated, whereas carnitine esters, citrate, glycerol-3-phosphate, and free fatty acid levels are reduced (36, 58–61). This lipid-rich state is also associated with obesity, as obese patients not only take in more dietary fatty acids than non-obese patients but also hydrolyze more stored fats from adipose tissue. As a result, the liver is exposed to very high fatty acid levels. The liver adjusts to this stress through adaptive metabolic changes (metabolic reprogramming), such as a shift to aerobic glycolysis and increased glutamine synthesis to provide α-ketoglutarate and citrate for the citric acid cycle, which collectively increase the risk of HCC (44). Obesity-mediated insulin resistance also promotes hyperinsulinemia, and oxidative and endoplasmic reticulum stress and changes in gut microbiota promote release of pro-inflammatory cytokines (62–68). The association between obesity and HCC suggests that

use of statins to treat obesity by inhibiting hepatic cholesterol biosynthesis and metformin to treat insulin resistance might offer some protection against HCC (odds ratios 0.74 and 0.38, respectively) (69, 70). FABPs, which regulate intracellular transport of fatty acids, are upregulated in a number of different cancers, including cancers of the bladder, breast, liver, lung, and prostate, and serve as biomarkers for cancer risk and aggressiveness (71). In a study of lipid-related HCC in a mouse model, Chiyonobu et al. showed that FABP4 was strongly upregulated in activated hepatic stellate cells (HSCs) resident within murine HCC tumors as well as in human metabolic-related HCC tumors but not in viral or alcohol-related HCCs, suggesting a mechanistic role of HSCs in NASH-related HCC (72).

LIPIDOMICS

Although a number of studies have examined changes in gene expression during HCC (73), fully understanding diseases as complex as NASH and HCC requires deep understanding of the internal state of individual cells. Single-cell transcriptomics is now possible; however, HCC tumor heterogeneity remains a challenge. A snapshot of the internal state can be partially reconstructed by assembling multiple complementary types of omics data. Lipidomics, first introduced in 2003, is the branch of metabolomics charged with characterizing the diversity of fatty acids and other lipid products within a cell, tissue, organ, or biofluid. Another goal of lipidomics is to uncover the enzymatic mechanisms and turnover kinetics responsible for changes in lipid metabolism (74). The lipidome is complex, diverse, and dynamic, representing tens to hundreds of thousands of molecular species in a constant state of flux (74). The following is an overview of how lipidomics can help elucidate the molecular mechanisms that contribute to HCC. The common lipidomics methods and selected lipidomics studies pertinent to HCC are summarized in Tables 1 and 2, respectively.

Several metabolomics and lipidomics studies involving HCC or NASH have been performed (Table 2). Puri et al. reported elevated levels of saturated and monounsaturated fatty acids (especially palmitoleic acid and oleic acid) and reduced levels of polyunsaturated fatty acids (e.g., linoleic acid) in patients with NAFLD or NASH compared to healthy controls (90). Patterson et al. compared plasma samples from HCC patients, healthy controls, and patients with cirrhosis or acute myeloid leukemia using ultra-performance liquid chromatography electrospray ionization-quadrupole mass spectrometry (UPLC-ESI-QTOFMS) and ultra-performance liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry (UPLC-ESI-TQMS) (91). They reported that glycodeoxycholate, deoxycholate 3-sulfate, bilirubin, biliverdin, and several fetal bile acids were elevated in the plasma of patients with HCC, whereas lysophosphocholine levels were reduced. Notably, they found that two very long chain fatty acids (VLCFAs), lignoceric acid and nervonic acid, were largely undetectable in plasma from HCC patients compared to patients with cirrhosis. Due to their extended length (≥22 carbons), VLCFAs are synthesized through a multistep elongase-dependent pathway in the endoplasmic reticulum and must be metabolized in peroxisomes (92).

TABLE 1 Common lipidomics methods and selected lipidomics studies involving HCC

Method	Description	HCC lipidomics studies
GC	Gas chromatography	(75) (76)
UPLC-MS	Ultra-high performance liquid chromatography mass spectrometry	(77)
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectrometry	(78)
ESI–MS	Electrospray ionization tandem mass spectrometry	(79)
IMS	Imaging mass spectrometry	(80)
LC-MS	Liquid chromatography mass spectrometry	(60, 81–83)
Shotgun lipidomics	Electrospray ionization mass spectrometry	(82)
RPLC-MS	Reversed-phase liquid chromatography mass spectrometry	(84)
LC/IT-TOF MS	Liquid chromatography/ion trap time-of-flight mass spectrometry	(85)
HPLC-MS	High-performance liquid chromatography mass spectrometry	(76, 86)
DESI-MSI	Desorption electrospray ionization mass spectrometry imaging	(87)
LC-ESI–MS	Liquid chromatography electrospray ionization mass spectrometry	(88)
UPLC-ESI-QTOF MS	Ultra-high performance liquid chromatography-electronic spray ionization-QTOF mass spectrometry	(89)
MALDI-FTICR MS	Matrix-assisted laser desorption ionization-Fourier transform ion cyclotron resonance mass spectrometry	(89)
GC-MS	Gas chromatography mass spectrometry	(83)

Lignoceric and nervonic acid, in particular, are involved in the maintenance of myelin, but VLCFAs are known to perform a range of functions, including skin barrier formation, sperm maturation, retinal functions, and liver homeostasis (92). VLCFAs also serve as precursors of inflammation-resolving lipid mediators, with potential roles in HCC formation (92). In a large case-control study comparing matched blood samples from patients before and after HCC diagnosis relative to healthy controls, Fages et al. identified a set of 16 metabolites involved in lipid and amino acid metabolism and ammonium detoxification that served as predictive biomarkers that differed between pre-diagnostic HCC patients and healthy controls, reflecting a characteristically altered metabolic state prior to HCC development (93). Two studies in China also reported panels of serum amino acid and fatty acid biomarkers able to predict HCC with area under the curve (AUC) greater than 0.96 (61, 94). Lin et al. recently showed that a decrease in palmitic acyl-based glycerophospholipids, a key component of the cell membrane, was associated with metastatic HCC (86).

TABLE 2 Selected lipidomics studies

Study	Description
Muir et al. (75)	The authors reported an increased ratio of long chain n6-polyunsaturated fatty acids to n3-polyunsaturated fatty acids in NASH and HCC using a Pten-null mouse model.
Weylandt et al. (76)	Using a fat-1 transgenic mouse model, the authors showed that increased omega-3 polyunsaturated fatty acids suppress HCC tumorigenesis by reducing inflammation.
Passos-Castilho et al. (77)	Serum ultra-high performance liquid chromatography mass spectrometry (UPLC-MS) lipid profiles discriminated patients with HBV-related HCC from patients with chronic HBV.
Passos-Castilho et al. (78)	Matrix-assisted laser desorption/ionization mass spectrometry lipid profiles discriminated patients with HCV-related HCC from patients with chronic HCV.
Krautbauer et al. (79)	Ceramide levels were found to be notably reduced in HCC tissues.
Morita et al. (80)	Levels of phosphatidylcholine containing palmitoleic acid or oleic acid were found to be elevated in HCC using imaging mass spectrometry.
Lu et al. (81)	Lipid signatures varied between HCC and serum samples. Plasmalogens (36:4) and (40:6) are potential serum biomarkers for HCC.
Zhou et al. (60)	Using liquid chromatography-mass spectrometry of serum samples, higher levels of long-chain acylcarnitines and lower levels of free carnitine and medium and short-chain acylcarnitines were detected in HCC.
Lu et al. (83)	Mass spectroscopic analysis of matched tissue and serum samples from patients with HCC was used to evaluate the usefulness of acetylcarnitine as a biomarker.
Chen et al. (85)	Ultra-fast LC/IT-TOF MS serum lipidomics was used to compare lipid profiles for patients with HBV, cirrhosis, and HCC. 75 out of 96 lipids were downregulated in patients with HCC compared to healthy patients.
Lin et al. (86)	Lipid profiling of HCC cells revealed anomalies affecting 93 different lipids. Reduced palmitic acyl glycerophosholipids were associated with greater metastatic activity.
Li et al. (89)	The number of polyunsaturated triacylglycerols with >2 double bonds was found to be reduced based on lipid profiling using UPLC-ESI-QTOF MS and MALDI-FTICR MS.

THERAPEUTIC ADVANCES TARGETING LIPID METABOLISM

Despite a better understanding of lipid metabolism, drugs targeting key steps in lipogenesis in various types of cancer are still in the experimental stage (Table 3) (95, 96). Given the central regulatory role of SREBPs in lipid metabolism, SREBPs represent promising drug targets. SREBP-1 and SREBP-2 are upregulated in glioblastoma and prostate cancer, respectively, and SREBP ablation or blocking has

TABLE 3 Drugs targeting lipid metabolism in tumor cells

Year	Compound	Target	Tumor Type	Model	Ref.
2019	Simvastatin	Lipid rafts	Human lung cancer	A549 cell	(101)
2018	Paclitaxel and vinblastine	Microtubule dynamics	Human osteosarcoma	U2OS cell	(102)
2017	Betulin	SREBPs	Human liver cancer	Diethylnitrosamine-injected mice model	(83)
2017	Cetuximab	Acetyl-CoA carboxylase (ACC)	Head and neck squamous cell carcinoma (HNSCC)	HN5, FaDu, Tu159, OSC19, MDA1986, UMSCC1, and Tu167)	(103)
2016	Nutlin-3 and actinomycin D	Ceramide synthase 6 (CerS6)	Human lung cancer	A549 cell	(104)
2015	TVB-2640	Fatty acid synthase (FASN)	-	Phase I	(105)
2014	Azoxymethane/ dextran sodium sulfate	Sphingosine-1-phosphate (S1P) lyase (SPL)	Colitis-associated cancer (CAC)	CAC murine model	(106)
2012	C75	FASN	Prostate cancer (PC)	LNCaP cell	(107)
2010	PX-866	PI3K	Human glioblastoma	U251, U87, LN229, and LN18 cells	(108)
2010	NDNSAs	Unknown	Human breast cancer	MCF-7 cell	(109)
2009	LCL385	Acid ceramidase (AC)	PC	PPC-1 cell	(110)
2009	15-dPGJ2	PPARγ	Colorectal cancer	CT-26 s.c. tumor model and an HL-60 xenograft model	(111)

been shown to induce cancer cell death and suppression of tumor growth (95). However, development of drugs that directly target transcription factors is difficult, and efforts have instead focused on drugs such as betulin, fatostatin, xanthohumol, and PF-429242 that inhibit the translocation of SREBP to the Golgi apparatus (95). Li et al. examined whether inhibition of ***de novo*** lipid biosynthesis is protective against HCC by blocking SREBP cleavage-activating protein in hepatocytes using betulin in a diethylnitrosamine-induced HCC mouse model (97). They found that blocking or ablation of this key component of the SREBP pathway suppressed HCC. Drugs have also been developed targeting specific steps in lipid metabolism. The ACC inhibitor GS-0976 has been found to reduce the extent of liver steatosis and fibrosis in NASH patients (98, 99), and drug targeting

of FASN has been shown to suppress HCC development (41, 53, 54). Several existing drugs such as statins and metformin are thought to have a protective effect against HCC (69, 70). Statins, as lipid-lowering agents, have long been used for the treatment of heart disease. They have also been reported to have a protective effect against tumorigenesis. Some published evidence supports the use of statins in HCC prevention in patients with liver disease (100).

CONCLUSION

While much progress has been made in limiting viral and environmental causes of HCC, new cases of NASH-related HCC are currently increasing and are likely to continue to increase for the foreseeable future. Many cases of NASH-related HCC are preventable through changes in lifestyle, including exercise and reduced intake of fructose and high-fat foods, but such changes are difficult to maintain. Therefore, there is an important unmet need to develop biomarkers to monitor changes in hepatic lipid metabolism in NAFLD patients, so that it is possible to intervene as early as possible in patients with the highest risk of progressing to NASH and cirrhosis. Early detection of HCC offers the best chance of treatment, while few procedures significantly improve survival in the case of advanced HCC. It is essential to determine the key molecular events that trigger hepatocarcinogenesis in order to facilitate drug development to prevent or slow development of HCC. Lipidomics provides a valuable tool to assess the detailed metabolic changes that may lead to initiation of liver cancer.

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6

Current Topics and Perspectives in Surgical Management of Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma is among the leading causes of cancer-related mortality. Due to the numerous surgical and non-surgical therapeutic options, the treatment strategy requires an optimal selection of patients based on tumor stage and liver functional reserve. A potentially curative surgical resection or liver transplantation is only recommended for patients with early stage disease. In this chapter, we overview the current topics and perspectives in the surgical management of hepatocellular carcinoma by disease stage with a special focus on new surgical techniques and expanding range of indications outside of the accepted Barcelona Clinic Liver Cancer algorithm.

Keywords: BCLC staging system; hepatocellular carcinoma; laparoscopic liver resection; liver transplantation; surgical treatment.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is among the leading causes of cancer-related death worldwide (1). Surgical resection or liver transplantation is the principal treatment option, depending on various factors, such as the liver functional reserve and tumor stage at the time of diagnosis. However, patients with HCC often present with late stage disease, which severely restricts the possibility for surgical resection. The Barcelona Clinic Liver Cancer (BCLC) staging system has become established as the most widely accepted staging system for HCC. In addition, the BCLC system is a clinical treatment guideline and comprises five stages related to the patient's performance, tumor condition, and liver functional reserve. Curative liver surgery is only recommended for early stages of HCC. In this chapter, we summarize the current role of surgical resection for HCC by disease stage in accordance with the BCLC treatment algorithm. Furthermore, we highlight the limitations of surgical resection and report data that support a treatment outside the accepted BCLC algorithm with a special focus for "advanced" but technically resectable HCC. Finally, we provide an overview on the ongoing developments of new surgical techniques, such as laparoscopic liver resection (LLR), robot-assisted liver resection (RALR), the associating liver partition, and portal vein ligation for staged hepatectomy (ALPPS) procedure, as well as perspectives in liver transplantation.

CURRENT SURGICAL MANAGEMENT OF HCC

Surgical resection or liver transplantation is the mainstay of potentially curative treatment for HCC. In addition to the surgical treatments or in patients with HCC who are not candidates for major liver surgery, there are various minimally invasive procedures besides systemic chemotherapy, including selective internal radiation therapy (SIRT), radiosurgery (Gamma Knife), transcatheter arterial chemoembolization (TACE), radiofrequency ablation (RFA), highly focused ultrasound (HIFU), microwave ablation (MWA), percutaneous ethanol injection (PEI), as well as irreversible electroporation (IRE). In this chapter, we focus on the current topics of surgical treatment for HCC at an early-, intermediate-, and advanced-stage based on the BCLC staging system. Furthermore, we discuss the current value of liver transplantation in the treatment of HCC.

Preoperative assessment

Staging of HCC is determined on the basis of size and number of tumors and the presence or absence of vascular invasion as well as extrahepatic lesions. The anatomic delineation of tumor extent is best achieved with dynamic multiphase computed tomography (CT), whereas the hepatic arterial phase is assessed separately from the portal venous phase with a late "wash-out" phase (2–4). Magnetic resonance imaging (MRI) appears to be more accurate in liver staging for HCC using multiphasic and multiparametric imaging by combining T1-, T2-, and diffusion-weighted imaging with dynamic multiphasic imaging (5–7).

The rate of extrahepatic disease spread of HCC at diagnosisis overall low, and the recognized sites of metastatic spread are lung, bone, peritoneum, and adrenal glands. Although not generally recommended, the 18-fluorodeoxyglucose positron-emission tomography (FDG-PET) can be used for the detection of otherwise occult distant metastatic disease (8). However, the risk of extrahepatic spread is higher in patients with a large tumor >5 cm, and such patients warrant additional imaging studies or staging laparoscopy with intraoperative ultrasound (IOUS) prior to surgical resection (9). Another benefit of IOUS is the identification of major intrahepatic vascular structures, which can be used to guide segmental or non-anatomic resections (10).

According to the BCLC staging system, liver function is assessed on the basis of the Child-Pugh classification, the presence of portal hypertension, and the presence of elevated serum bilirubin concentrations (11). A scoring system for assessing the severity of chronic liver disease, and subsequently in prioritizing for receipt of a liver transplant, is the Model for End-Stage Liver Disease or MELD (12). Currently, this score is used by the United Network for Organ Sharing (UNOS) and Eurotransplant for prioritizing allocation of liver transplants (12, 13). The clearance of indocyanine green (ICG-15) at 15 min can be used as a defining criterion for the selection of patients as well as liver resection type (14, 15). Moreover, the newly developed LiMAx® (Humedics, Berlin, Germany) test, a ^{13}C-labelled methacetin-based metabolic liver function capacity test, is a suitable diagnostic tool to predict the individual risk of postoperative liver failure after liver surgery (16).

BCLC staging classification

Since the staging system was first published in 1999, the BCLC staging system has emerged as a primary system for staging as well as a clinical guideline for the treatment of HCC (17). The BCLC staging system stratifies treatment algorithms based on the patient's performance status, the size and number of tumor nodules present, the presence of liver impairment, including portal hypertension, as well as degree of cirrhosis as measured by the Child-Pugh classification (Figure 1). The classification has been updated according to evidence-based data and is presently endorsed as the standard system for HCC management by the American Association for the Study of Liver Disease, American Gastroenterology Association, European Association for the Study of Liver and the European Organization for the Research and Treatment of Cancer (18–20). Nonetheless, the BCLC system has been heavily criticized for its extremely limited criteria for resection and its recommendations against potentially curative liver surgery for "advanced" but technically resectable HCC.

Early-stage disease

According to the BCLC algorithm, only patients with very early stage disease (BCLC 0), with a single lesion less than 2 cm, no evidence of portal hypertension and normal bilirubin levels, are recommended to undergo liver resection. Patients with early-stage disease (BCLC A), defined as single or three nodules less than 3 cm, fulfill the Milan criteria and are recommended to undergo liver

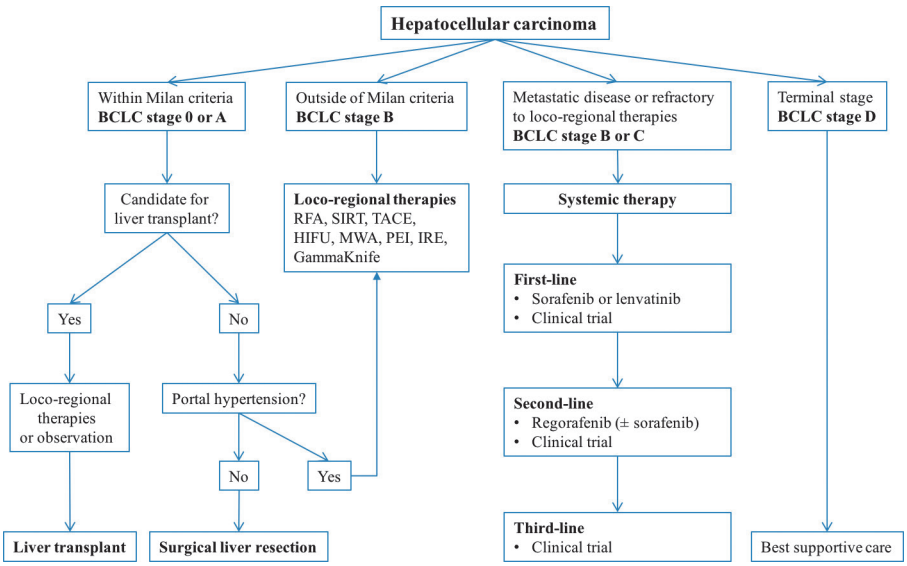

Figure 1 Treatment algorithm for hepatocellular carcinoma (1).

transplantation unless other comorbidities are present (21). Currently, there is an ongoing debate regarding surgical resection versus liver transplantation for (very) early-stage HCC. Concerning this matter, patients with preserved liver function and low-level cirrhosis have similar survival outcomes after liver surgery compared to liver transplantation (22). Due to low availability of organs, lifelong immunosuppression, and larger healthcare costs, orthotopic liver transplantation should be employed for patients with more severe cirrhosis (23).

According to the BCLC algorithm, PEI and RFA are recommended for early-stage disease. However, numerous studies revealed an improved survival outcome in patients who underwent surgical resection (24–26). A meta-analysis including 21,000 patients demonstrated a better overall survival as well as recurrence-free survival after surgical resection in comparison with RFA and/or PEI (24). Xu et al. confirmed this finding in another meta-analysis of over 2,500 patients (25). In addition, a prospective randomized trial including 235 patients who met Milan criteria showed a 5-year overall survival of 76.65% in the surgery group versus 54.78% in the RFA group (26). However, due to small residual liver volume, low liver functional reserve, or poor performance status, only 10–35% of patients with very early- and early-stage disease underwent liver resection (19, 27). Nonetheless, these study data underline the advantages of surgical liver resection compared to local ablative therapies. Therefore, liver surgery should be offered in those patients with an early-stage HCC who can tolerate a major hepatic resection based on the underlying liver disease as well as comorbidities. Liver transplantation should be performed in patients with HCC and patients with life-limiting cirrhosis. Otherwise, in patients with poor performance, local ablative therapies should be considered (23).

Intermediate-stage disease

Intermediate-stage disease is defined by the BCLC staging system as patients with multinodular HCC and good performance status as well as no clinical evidence of portal vein invasion, nodal disease, or extrahepatic metastases. Patients with BCLC stage B are recommended for TACE, but several studies support surgical liver resection in intermediate stage HCC (28–31). A retrospective analysis of 393 patients by Zhong et al. confirmed a statistically significant improvement of median overall survival of patients who underwent surgical resection (59% vs. 29% at 3 years) compared to patients who underwent TACE (28). Another study from Ho et al. including 1,065 patients with multiple HCCs confirmed a better 5-year survival rate (36.6% vs. 11%) in the liver surgery group compared to the TACE group (30). Furthermore, a prospective analysis of 168 patients with multiple HCC lesions greater than 5 cm showed the best 5-year survival of 50.5% in patients who responded to neoadjuvant TACE followed by surgical liver resection (31). This finding was confirmed by a retrospective cohort study involving 110 patients (32). The median survival of patients who underwent TACE followed by liver resection was 47 months compared to 20 months in patients who received TACE alone. To summarize, a treatment strategy of TACE for downstaging followed by surgical liver resection seems to be beneficial for patients with large and multifocal, but resectable, HCCs falling within BCLC stage B.

Advanced-stage disease

The BCLC defines advanced-stage disease (stage C) as HCC with nodal and portal vein involvement, extrahepatic spread, or patients with poor performance status. According to the BCLC algorithm, the treatment of patients with stage C HCC is systemic sorafenib therapy (33). At present, systemic treatment of HCC is evolving rapidly, and three new multikinase inhibitors (i.e., regorafenib, lenvatinib, and cabozantinib) have been shown to be effective in phase 3 clinical trials (34). In the REFLECT trial, lenvatinib has shown to be non-inferior to sorafenib in a front-line setting (35). The treatment sequence of sorafenib plus regorafenib showed an important extension in overall survival of patients with advanced HCC, in the second-line setting (36). Based on the findings of CELESTIAL phase 3 trial, a treatment with cabozantinib resulted in longer overall survival and progression-free survival than placebo (37). Thus, the multikinase inhibitor cabozantinib seems to be an additional treatment option for use in adults with advanced HCC previously treated with sorafenib. Notably, sorafenib demonstrated no benefit in the adjuvant setting in HCC following surgical resection or local ablation (38). To date, no new drugs are approved for the adjuvant setting (39). However, there are numerous retrospective studies supporting surgical resection in patients with advanced HCC (40–46). A retrospective study by Ruzzenente et al. showed a statistically significant longer median survival (27 months vs. 12 months) in HCC patients with macroscopic vascular involvement who underwent liver surgery compared to systemic therapy only (41). A combination of local ablation via RFA or TACE and surgical resection seems to be a treatment option in patients with bilobar

HCC metastases, but preserved liver function (44). This finding was confirmed by Liu et al. by a better survival outcome in selected patients with bilobar metastases and satisfactory liver function who underwent a combination of hepatic resection and ablation compared to non-resectional therapies (45). Generally, in areas with high incidence of HCC, such as Asia, surgical resection is commonly offered to patients with stage C disease (46, 47).

In summary, the retrospective literature highlights the value of a combined treatment strategy involving surgical liver resection and local ablation in a selected patient population of advanced HCC, particularly when liver function is preserved. Nonetheless, the survival of advanced HCC is still poor, and prospective randomized controlled studies are needed to obtain data with higher quality in matters of multimodality treatment.

Liver transplantation

The only potentially curable treatment of HCC is orthotopic liver transplantation, which allows not only the cure of the HCC but also the treatment of the underlying liver disease (48). However, due to the strict Milan criteria of liver transplantation in HCC patients (which include patients with one tumor <50 mm or up to three tumors <30 mm) and the low availability of organs, only a small number of patients receive a liver transplant (21). Even though a 4-year survival rate of patients within Milan criteria is reported to be over 70% (21), approximately half of the patients develop liver cirrhosis post-transplantation (49). Nonetheless, a recent systemic review of 90 studies including 17,780 patients over a 15-year period confirmed the Milan criteria as major determinants of the prognosis of patients undergoing orthotopic liver transplantation for the treatment of HCC (50).

Limitations of liver surgery

Surgical liver resection should be performed in patients with HCC that is amenable to a negative resection margin (R0) and in patients with good liver functional reserve (23). Moreover, there is a limitation of resection by the need to maintain an adequate future liver remnant of commonly quoted 20% (volumetric prediction) in patients without pre-existing liver dysfunction (51). Remarkably, the accepted future liver remnant values are more conservative in patients after chemotherapy treatment (30%) and in patients with evidence of cirrhosis (40%) (52). Therefore, these patients need a more conservative approach due to lower functional liver reserve. Patients with advanced cirrhosis or portal hypertension may be better managed by liver transplantation or ablative therapies. However, the risk of hepatic resection must be balanced by the patient's potential benefit from aggressive surgery.

PERSPECTIVES IN SURGICAL MANAGEMENT OF HCC

In the last two decades, there has been continuous development of new surgical techniques allowing for safer and more aggressive liver resections. Moreover, surgeons have developed a deeper understanding of physiology and functional

reserve of the liver. Due to the broad criticism of the BCLC guidelines for recommending non-surgical treatment, expansion of surgical management for HCC should be explored. Here, we review significant advancements in surgical management of HCC including new techniques, particularly LLR, RALR, and the ALPPS procedure. Moreover, we recap extended criteria for liver transplantation beyond the Milan criteria.

Laparoscopic liver resection

Since the first use of laparoscopy for liver surgery in 1991 (53), several studies have shown the safety and efficacy of LLR with many advantages, including reduced blood loss and shorter hospital stay (54, 55). In addition, the International Survey on Technical Aspects of Laparoscopic Liver Resection (INSTALL) study revealed an increased number of LLR cases worldwide (56). The Second International Consensus Conference for Laparoscopic Liver Resection was held in Morioka, Japan, in 2014,with the dual goal of defining the current role of LLR and developing recommendations and guidelines (57). Despite a lack of high-level studies, minor LLR has become a standard practice. In contrast, the recommendation of the consensus meeting for major LLR was that surgeons undertaking these procedures should be experienced in both the liver surgery and advanced laparoscopy due to remaining risks associated with the newness of the procedure (57, 58). Furthermore, postoperative outcomes should be evaluated by randomized controlled trials and in registries.

In terms of the oncological outcome, several retrospective studies as well as meta-analysis demonstrated that LLR is non-inferior to laparotomy with fewer adverse effects, smaller amounts of blood loss, and shorter hospital stay (59–64). No significant differences between open and laparoscopic liver surgery were observed regarding overall survival of patients with early-stage HCC. Moreover, LLR seems to be superior in patients with impaired liver function (65, 66), and a better disease-free survival rate in advanced HCC patients was observed (67). Currently, a novel scoring system of surgical difficulty based on tumor factors (including location and relationship to large vessels) and liver functional reserve has been proposed as a training guideline (68, 69). In addition, this novel scoring model has been correlated with the postoperative outcome (70, 71). In summary, the laparoscopic approach will lead to expanding the surgical indications for HCC, especially in patients with chronic liver disease. Furthermore, a step-by-step training system for surgeons based on the novel difficulty scoring system can make this expansion safer and more effective for patients with HCC.

Robot-assisted liver resection

Since its inception in 2002, the innovative approach of RALR has gained worldwide acceptance (72–74). The indications for robotic liver resections are similar to those of LLRs, according to the Morioka consensus (57). In general, the indications are solitary lesions <5 cm and located in liver segments 2–6 (75). Nonetheless, a number of reviews revealed extended indications for the robotic approach including every segment of the liver (73, 74, 76). Contraindications

are tumors with the invasion of major vascular structures or patients who are pneumoperitoneum-intolerant. A study by Lai et al. confirmed no significant differences in oncological outcomes between the robot-assisted and the conventional laparoscopic approach (77). Moreover, both techniques are similar in terms of blood loss, morbidity, and hospital stay, but prolonged operative times and increased costs were more evident in the robotic approach (76). The major advantage of RALR may lie in sectoral, segmental, or subsegmental resections in difficult-to-reach positions like posterior–superior segments and caudate lobe (78). Another benefit is the possibility of a shortened learning curve for complex liver resections based on the experiences in robot-assisted pancreatic resection (79). According to the currently available literature, RALR seems to be safe and feasible in selected patients with HCC. However, more prospective randomized studies are needed to determine the exact role of RALR within the treatment algorithm of HCC.

Associating liver partition and portal vein ligation for staged hepatectomy

The ALPPS procedure or "*in situ* split liver resection" is a novel two-stage surgical approach to induce rapid hypertrophy of the future liver remnantin a short period of time (80). The procedure is based on a combination of transection of the liver along the falciform ligament and dissection of the right portal veinin order to induce hypertrophy in the future liver remnant in patients undergoing an extended right hepatectomy. The ALPPS procedure might be considered in the following clinical scenarios: involvement of the right portal vein by HCC, progressive HCC with high risk for tumor progression between two stages of conventional surgical approach, and progressive HCC with extension to the vena cava or right heart atrium. Contraindications for ALPPS include inoperable hepatic metastasis in the future liver remnant, significant portal hypertension, and unresectable extrahepatic metastasis (81).

Nonetheless, ALPPS is associated with several adverse effects, including biliary leakage and intraperitoneal infection (82). An overall mortality rate of 59–64% has been reported in association with ALPPS (83). According to the international ALPPS registry, the overall 90-day mortality rate was 8.8%, in which 75% of deaths were related to postoperative liver failure. Moreover, patients with a model of end-stage liver disease (MELD) score of more than 10 showed a significantly increased mortality (84). For this reason, controversy exists regarding the use of ALPPS in real clinical practice, and patients should be carefully evaluated and selected in order to avoid postoperative small-for-size syndrome or acute liver failure (85). However, the evidence of oncological endpoints as well as technical availability of ALPPS is scarce up to now, and recent studies reported a perioperative mortality rate of 31% for HCC patients (86). In summary, the ALPPS procedure should be considered only in a highly selected patient population and should only be performed in highly specialized centers for liver surgery. Further studies are needed to determine the criteria for use of ALPSS and to define its value compared to other treatment algorithms of HCC.

Perspectives in liver transplantation—extended criteria

Since its establishment by Mazzaferro et al. in 1996 (21), the Milan criteria have been applied widely around the world in the selection of patients for orthotopic liver transplantation. However, the Milan criteria are very restrictive concerning post-transplant recurrence rates and could be expanded, as long as patient outcome is not impaired. The University of California San Francisco (USCF) criteria are the most widely accepted for the expansion of the Milan criteria: a solitary tumor ≤65 mm, or two to three tumors ≤45 mm, and total tumor diameter ≤80 mm, without vascular invasion or distant metastasis (87). According to Yao et al., the USCF 1-year and 5-year survival rates from lifetime data of 70 patients over a 12-year period were 90 and 75%, respectively (87). Moreover, the disease recurrence rates were comparable to those of the Milan criteria (88). Currently, further extended criteria for the selection of patients with HCC beyond the Milan criteria are subject of ongoing research. Assessment of the two clinical biomarkers, alpha-fetoprotein (<200 ng/mL) and des-gamma carboxyprothrombin (<400 mAU/mL), showed an improved selection of patients with HCC for liver transplantation (89–91). A study by DuBay et al. confirmed excellent post-liver-transplantation survival rates of patients with any HCC size and number, when an aggressive bridge-to-transplant therapy was applied and a poorly differentiated tumor was ruled out by liver biopsy (92). Tumor growth beyond the acceptable size can cause a drop out from the waiting list for transplantation. Importantly, liver resection prior to transplantation does not increase the morbidity or impair long-term survival following liver transplantation in selected patients (93).

However, excessive expansion of inclusion criteria will result in an increase in waiting time and a deterioration of survival among patients on the waiting list (94). Thus, the decision for a liver transplantation beyond the Milan criteria should be based on a case-by-case consideration, balancing the operative risk versus the potential survival benefit. Moreover, liver resection should be considered as a bridge-to-transplant option in highly selected patients with HCC.

CONCLUSION

HCC is a tumor with highly variable biology that often occurs in the setting of chronic liver disease. Patients often present with late stage disease, which excludes surgical resection from the treatment options. In this chapter, we have highlighted the current topics and perspectives in surgical management of HCC. Treatment strategies require optimal selection of therapies based on various tumor factors and liver functional reserve. The introduction of new surgical techniques, especially the laparoscopic approach; the combination of surgery with ablative therapies; and the expansion of indications for surgery beyond the conservative BCLC algorithm as well as beyond the Milan criteria have increased the variety of surgical treatment options for carefully selected patients with HCC. However, regarding the complexity of all treatment options, more detailed, rigorous studies are needed to determine evidence-based guidelines.

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7

Tyrosine Kinase Inhibitors in the Treatment of Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma is the third leading cause of cancer-related mortality in the world. Locoregional therapy is used for early stage hepatocellular carcinoma. Tyrosine kinase inhibitors have been the mainstay of treatment for advanced hepatocellular carcinoma. Sorafenib was the first drug approved based on data from two pivotal phase III trials. Although sorafenib provided a survival benefit, development of adverse events limits its use in some patients. These adverse events, such as hand–foot syndrome and diarrhea, have a significant impact on the quality of life and, in some circumstances, are severe enough to prompt cessation of the drug. In recent times, a range of new therapeutic options have come on the scene including lenvatinib, regorafenib, and cabozantinib. Lenvatinibis now approved as an alternative first-line agent for hepatocellular carcinoma. Regorafenib and cabozantinib are both second-line agents. These medications provide a promising range of treatment options for patients who progress on sorafenib or are intolerant to it. This chapter provides an insight into the range of tyrosine kinase inhibitors available for the treatment of hepatocellular carcinoma.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancer and is the third leading cause of cancer-related mortality in the world. Despite advances in treatment, 5-year survival rates are still poor at 18% (1). Unfortunately, up to 80% of patients present with advanced, incurable disease (2). The Barcelona Clinic Liver Cancer (BCLC) algorithm was published in 1999 and is the most widely used staging system. There are other staging systems in use such as the Hong Kong Liver Cancer staging system but these are not as commonly applied. BCLC guidelines classify patients with preserved liver function who have macrovascular invasion or extrahepatic spread of disease and Eastern Cooperative Oncology Group performance status (ECOG) 1–2 as having advanced stage disease (Stage C) (3) (Figure 1). In this group of patients, systemic therapy is recommended (3). Prior to 2007, there was a lack of effective treatment options for patients with advanced HCC. Traditional chemotherapeutic agents were non-targeted and resulted in significant side effects due to their widespread cytotoxic or cytostatic mechanisms of action. It was evident that therapies such as doxorubicin and FOLFOX (fluorouracil, leucovorin, oxiplatin) had insufficient antitumoral activity and caused excessive toxicity in the context of cirrhosis. As a result, the ongoing development of systemic therapies is centered on the development of more targeted systemic therapies. The realm of systemic therapy for HCC is rapidly evolving and encompasses a range of drugs such as tyrosine kinase inhibitors (TKIs), monoclonal antibodies (ramucirumab), and immune check point inhibitors (nivolumab and pembrolizumab). As the focus of this chapter is TKIs (Table 1), other systemic therapies will not be discussed here. The authors recommend referring to "Immune checkpoint

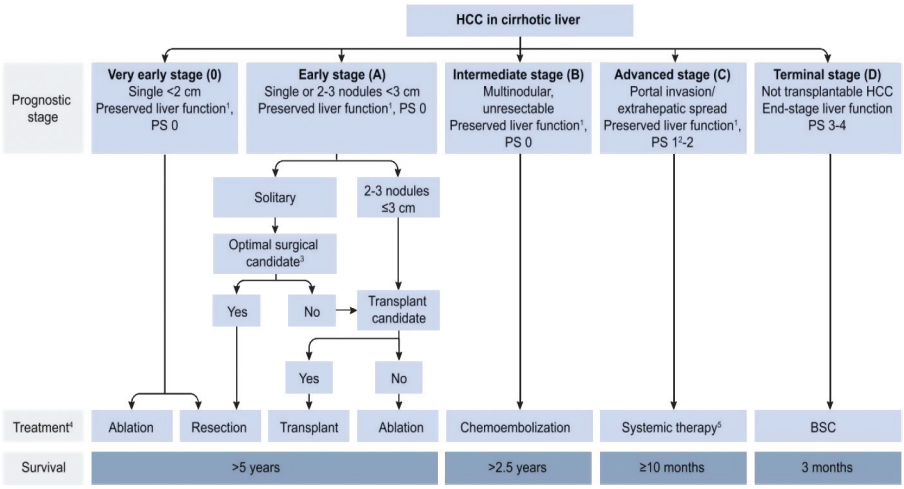

Figure 1 BCLC staging system and treatment strategy (3).

TABLE 1 Summary of tyrosine kinase inhibitors and relevant trials

Summary of tyrosine kinase inhibitors

TKI	Target receptors	Studies	Year	Type	No. of patients	Outcome	Current use
Sorafenib	VEGFR2, VEGFR3, PDGFR, c-kit, FLT-3, RET	SHARP Trial	2006–2008	Phase 3 randomized double-blind placebo-controlled trial (sorafenib vs. placebo)	602	Improved median OS (10.7 vs. 7.9 months, HR 0.69)	First-line agent for advanced HCC
		Asia Pacific Trial	2005–2007	Phase 3 randomized double-blind placebo-controlled trial (sorafenib vs. placebo)	226	Improved median OS (6.5 vs. 4.2 months, HR 0.68)	
Lenvatinib	VEGFR 1–3, FGFR 1-4, PDGF-alpha, RET, KIT	Ikeda et al	2010–2011	Phase 2 single-arm open label multicenter study	46	Improved median OS 18.7 months (95% CI: 12.7–25.1) TTP 7.4 months (95% CI: 5.5–9.4)	First-line agent for advanced HCC
		REFLECT Trial	2013–2015	Phase 3 open-label multicenter non-inferiority study (lenvatinib vs. sorafenib)	954	Improved median OS 13.6 months for lenvatinib versus 12.3 months for sorafenib (HR 0.92) TTP 7.4 months lenvatinib vs. 3.7 months sorafenib (HR 0.66)	
Regorafenib	VEGFR 1–3, oncogenic tyrosine kinase receptor	RESOURCE Trial	2013–2015	Phase 3 randomized double-blind parallel group (regorafenib vs. placebo)—previously treated with sorafenib	573	Improved median OS (7.8 vs. 10.6 months, HR 0.63) Improved PFS (1.5 vs. 3.1 months, HR 0.46)	Second-line agent for advanced HCC previously treated with sorafenib
Cabozantinib	MET, VEGF, AXL, RET, ROSI, TYRO3, MER, KIT, TRKB, FLT3, TIE2	CELESTIAL Trial	2013–2017	Phase 3 randomized double-blind placebo-controlled trial (cabozantinib vs. placebo)	707	Improved median OS (8 vs. 10.2 months, HR 0.76) Improved PFS (1.9 vs. 5.2 months, HR 0.44)	Second-line agent for advanced HCC previously treated with sorafenib

CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression-free survival; TTP, time taken to progression.

inhibition: Prospects for prevention and therapy of hepatocellular carcinoma" by Elsegood et al. for further information (4). Tyrosine kinases are involved in the activation of a wide range of proteins by phosphorylation. TKIs bind to the active site of tyrosine kinases, thus preventing phosphorylation and inhibiting downstream signal transduction of a range of growth factors. By blocking the key tyrosine kinase pathways in cancers such as the vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor 2(EGFR), and platelet-derived growth factor (PDGFR), tumor growth is halted.

SORAFENIB

Sorafenib was the first TKI to receive approval from the Food and Drug Administration (FDA) for systemic treatment of HCC in 2007 and remains the first-line therapy. It is an oral multi-kinase inhibitor that targets VEGFR2, VEGFR3, PDGFR, c-kit, FLT-3, and RET (5). This in turn prevents tumor angiogenesis and tumor cell proliferation, increasing the rate of apoptosis. The Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) study and the Asia-Pacific trial were the two major trials which proved the efficacy of sorafenib.

The SHARP study was a phase III, randomized, double-blind, placebo-controlled trial carried out between 2006 and 2008 (5). It was conducted across multiple centers in North America and Europe. Child-Pugh (CP) A patients with advanced HCC who had not previously had any systemic treatment were recruited for the trial. For trial inclusion, the patients were required to have adequate hepatic, renal, and hematological reserve, and an ECOG of 0–2. 602 patients were included and randomized in a 1:1 ratio to oral sorafenib 400 mg twice a day versus placebo.

The Asia Pacific trial was also a multinational, phase III, randomized, double-blind placebo-controlled trial (6). It had similar inclusion criteria to the SHARP trial but was carried out in the Asia Pacific region; 226 patients were enrolled and randomized in a 2:1 ratio to sorafenib 400 mg twice a day versus placebo. Both trials permitted dose reductions in treatment interruptions in the event of drug toxicity.

The SHARP study demonstrated an improvement in overall survival of 2.8 months in favor of sorafenib (10.7 vs. 7.9 months, hazard ratio [HR] 0.69) (5). This effect was also seen in the Asia Pacific trial with sorafenib improving overall survival from 4.2 to 6.5 months (HR 0.68) (6). The difference in median survival between the two trials can be attributed to differences in the study populations. The patients in the Asia-Pacific trial were younger (51 years old vs. 65 years old), had predominantly Hepatitis B-related disease (75% vs. 18%), and had more advanced disease with more extrahepatic spread.

Dose reductions due to adverse events (AE) were common across both trials (26% in SHARP, 30.9% in Asia Pacific trial) (5). The most common TKI-associated AEs and their relevant management strategies are discussed later in this chapter. The median duration of treatment was only 5.3 months in the SHARP trial (5). Unfortunately, tolerability of sorafenib impacted on the

duration of treatment and hence survival. A pooled analysis of the SHARP and Asia Pacific trials was undertaken to determine the predictors of sorafenib benefit. It was found that patients who developed early dermatological AEs (within the first 60 days) had a better median overall survival than those who did not (18.2 vs. 10.1 months) (7).

With the widespread usage of sorafenib, there is now real-world data available for comparison with the two phase III trials. The Global Investigation of Therapeutic Decisions (GIDEON) study was a large prospective observational registry with 3,371 patients to evaluate the safety and usability of sorafenib in the HCC population. This cohort demonstrated that a higher CP score and a higher BCLC stage were associated with a shorter median survival—CP A: 13.6 months, CP B: 5.2 months, and CP C: 2.6 months (8). It also showed that the overall incidence of AE was comparable between CP A and B patients (8). A Taiwan-based study by Huang et al. used sorafenib in a broader HCC population, including patients who were CP B and CP C. They reported median overall survival rates of 8 months (9).

As there is significant genetic heterogeneity in HCC, this can result in both primary and secondary loss of response to sorafenib. Other therapeutic options are required for patients who have lost response. The benefits of sorafenib in combination with other therapies are being investigated. There have been trials combining sorafenib with doxorubicin, and radioembolization with Yttrium90 and erlotinib. At present, none of these trials have shown an improvement in median overall survival (10–13). The STORM trial was a phase III study comparing adjuvant sorafenib to placebo after radiofrequency ablation or hepatectomy. The use of sorafenib did not result in an improvement in recurrence-free survival (14). Further research into combination therapies that include sorafenib is required to provide advanced HCC patients with more therapeutic options.

LENVATINIB

Sorafenib, which was shown to improve survival in the SHARP and Asia-Pacific trials, has been the standard first-line therapy for unresectable HCC since 2007. Since then, other molecular-targeted agents have been developed and tested in clinical trials. However, this has been marked by four failed phase III trials evaluating sunitinib, brivanib, linifanib, and erlotinib plus sorafenib that did not show non-inferiority or superiority to sorafenib in terms of overall survival in the first-line treatment of HCC (10, 15–17). These negative trials created a need to develop new drugs as first-line agents for the effective management of HCC.

Lenvatinib was discovered at Tsukuba Research Laboratory in Japan as a result of research on angiogenesis inhibitors. It is an oral multikinase inhibitor that targets VEGF receptors 1–3, FGF receptors 1–4, PDGF receptor α, RET, and KIT and is an extremely effective inhibitor of tumor angiogenesis (18). It has shown activity against a range of solid tumors. Lenvatinib monotherapy is approved for the treatment of radioiodine-refractory differentiated thyroid cancer (19). With everolimus, it is used as a combined treatment for advanced renal cell carcinoma following one previous antiangiogenic therapy (20). The studies proving efficacy of lenvatinib in advanced HCC are described below.

A phase II trial in patients with HCC conducted in Japan and South Korea confirmed the potent antitumor effect of lenvatinib and the feasibility of managing AEs in patients with HCC (21). It was a phase II, single-arm, open-label multicenter study which was conducted on 46 patients between July 2010 and June 2011 with advanced HCC who did not qualify for surgical resection or local therapies. The patients received a dose of 12 mg once daily in 28-day cycles. The median time to progression was 7.4 months (95% CI: 5.5–9.4). The median overall survival was 18.7 months (95% CI: 12.7–25.1). Seventeen patients (37%) had partial response and 19 patients (41%) had stable disease (20). The most common any-grade AEs were hypertension (76%), palmar–plantar erythrodysesthesia syndrome (65%), decreased appetite (61%), and proteinuria (61%). Dose reductions and discontinuations due to AEs occurred in 34 (74%) patients and 10 (22%) patients, respectively (20). Dose reductions were needed more often in patients with a low body weight. A later detailed analysis of the pharmacokinetics of lenvatinib in patients with HCC determined that the optimal dose was 8 mg/day for patients weighing less than 60 kg and 12 mg/day for patients weighing 60 kg or more. These findings paved the way for the phase III trial named the REFLECT study.

The REFLECT trial was an open label phase III, multicenter non-inferiority trial which enrolled patients with unresectable HCC who had not received prior systemic chemotherapy. This was conducted at 154 sites across 20 countries. Stratification factors included region (Asia or non-Asia), macroscopic portal vein involvement and/or extrahepatic spread, ECOG performance status (0 or 1), and body weight (<60 kg or ≥60 kg); 954 eligible patients were randomized in a 1:1 ratio to lenvatinib (12 mg daily for body weight ≥60 kg, and 8 mg daily for body weight <60 kg) or sorafenib (400 mg twice daily for all patients) arms. Treatment was continued until disease progression or occurrence of intolerable adverse event. The primary endpoint was overall survival, measured from the date of randomization until the date of death from any cause (22).

Secondary endpoints included evaluation of progression-free survival, time to progression, objective response rate, quality-of-life measurements, and plasma pharmacokinetics lenvatinib exposure parameters.

Lenvatinib was non-inferior to sorafenib revealing that the median survival time for lenvatinib was 13.6 months (95% CI 12.1–14.9), and for sorafenib, it was 12.3 months (95% CI 10.4–13.9). The objective response rate for lenvatinib was higher (24% vs. 9%), and the median time to progression was longer (7.4 vs. 3.7 months, HR = 0.66, 95% CI 0.57–0.77) (22).

From the AEs point of view, the rate of grade 3 or 4 hypertension was higher with lenvatinib (23% vs. 14%), while the hand–foot skin reaction was more frequent with sorafenib (52% vs. 37%any grade, and 11% vs. 3% grade 3 or worse), as was alopecia of any grade (25% vs. 3%) (22).

Based on the REFLECT study, lenvatinib was approved in Japan in March 2018 for the treatment of unresectable HCC. In August 2018, it received approval in the United States adding another agent to the arsenal of medications used as the first-line treatment of HCC. Consensus-based guidelines from the National Comprehensive Cancer Network (NCCN) suggest limiting the use of lenvatinib to individuals with CP A cirrhosis. Therapeutic combinations involving lenvatinib with an immune checkpoint inhibitor have potential as future treatment strategies (23).

REGORAFENIB

Regorafenib is an oral diphenylurea multikinase inhibitor that targets kinases involved in tumor angiogenesis, cell proliferation, and survival (24, 25). Its chemical structure is similar to that of sorafenib, differing only in the presence of an extra fluorine atom. This change in structure is theorized to provide a wider range of targets to inhibit (25). Regorafenib was initially used for the treatment of metastatic colorectal cancer and gastrointestinal stromal tumors. In 2017, regorafenib received FDA approval for the treatment of patients with advanced HCC who had previously been treated with sorafenib. Despite this, regorafenib is currently not approved by the Therapeutic Goods Administration in Australia.

The RESORCE study was the first successful randomized, double-blind, parallel group, phase III trial for regorafenib. It was a multicenter trial conducted across 152 sites in 21 countries; 573 patients were enrolled and randomized in a 2:1 ratio to regorafenib 160 mg or placebo for the first 3 weeks out of every 4-week cycle. Both groups received best supportive care (26). The study included CP A patients with BCLC stage B or C disease who had documented progression of their disease on imaging despite sorafenib. Patients must have been able to tolerate sorafenib at a dose of at least 400 mg daily for 20 out of 28 days (26). Exclusion criteria were intolerance of sorafenib and failure of previous systemic therapy.

The study's primary endpoint was overall survival, defined as time from randomization to death from any cause. Secondary endpoints were progression-free survival (based on radiological or clinical data), time to progression, objective response (complete or partial response), and disease control rate (defined as complete response, partial response, or stable disease for >6 weeks based on mRECIST criteria). Treatment was continued until progression, death, or unacceptable toxicity from the drug.

The RESORCE trial showed that regorafenib increased survival, as compared with placebo, from 7.8 to 10.6 months (HR = 0.63, $P < 0.0001$). Progression-free survival in patients on regorafenib also increased from 1.5 to 3.1 months (HR = 0.46, $P < 0.0001$); 54% of patients had stable disease with 23% experiencing a progression in their disease (26). Subanalysis showed that patients treated with sorafenib and regorafenib had a longer survival time of 26.0 versus 19.2 months in patients who had sorafenib and placebo (27).

Of note, 93% of patients on regorafenib experienced an adverse event with up to 50% having a severe (Grade 3 or 4) adverse event. This would have resulted in interruption to treatment and dose reduction. As the duration of dose interruption and degree of dose reduction were not formally reported in the results of the trial, it is difficult to ascertain its effect on the results.

Real-world data are available since the RESORCE trial. A retrospective multicenter study in Japan by Ogasawara et al. has verified the safety and efficacy of the medication in advanced HCC. The median progression-free survival is comparable to that of the RESORCE trial. The Japanese study did have a significantly longer median overall survival of 17.3 months (28). However, this difference may be attributable to the use of other systemic therapies such as lenvatinib in the patients who discontinued regorafenib.

Based on the existing data, regorafenib is a suitable second-line therapy for advanced HCC in patients who tolerated sorafenib, even if they experienced progression. It is yet unclear if it would be appropriate to use in a sorafenib naïve population.

CABOZANTINIB

Cabozantinib is another oral multikinase inhibitor targeting multimodal pathways. Cabozantinib was evaluated for its inhibitory activity against a variety of kinases and was identified as an inhibitor of MET (hepatocyte growth factor receptor protein) and VEGF receptors. In addition, cabozantinib inhibits other tyrosine kinases including the GAS6 receptor (AXL), RET, ROS1, TYRO3, MER, the stem cell factor receptor (KIT), TRKB, Fms-like tyrosine kinase-3 (FLT3), and TIE-2 (29, 30). MET and AXL genes are associated with poor prognosis and development of resistance to VEGF inhibition. Thus, developing inhibitors that simultaneously inhibit VEGF and other pathways involved in tumor invasion and metastasis may confer broad and potent antitumor efficacy. Cabozantinib was initially indicated for the treatment of advanced renal cell carcinoma in treatment naïve adults with intermediate or poor risk or in adults following prior treatment with VEGF-targeted therapy.

Efficacy of cabozantinib in treating advanced HCC was shown in phase III CELESTIAL trial (31). It was a randomized double-blind multicenter study conducted across 95 centers in 19 countries. A total of 707 patients were enrolled and randomized in 2:1 ratio to cabozantinib 60 mg or placebo. Eligible patients had received previous treatment with sorafenib, had evidence of disease progression after at least one systemic treatment, and could have received up to two previous systemic treatments for advanced HCC. The patients were 18 years of age or older who had received a pathological diagnosis of HCC not amenable to curative treatment and had CP A cirrhosis. Furthermore, patients were required to have an ECOG score of 0 or 1. Exclusion criteria included previous treatment with cabozantinib and uncontrolled clinically significant illness.

The trial's endpoint was overall survival defined as the time from randomization to death from any cause. Secondary endpoints were progression-free survival defined as the time from randomization to radiographic progression or death from any cause whichever occurred first and objective response rate (the percentage of patients with a confirmed complete or partial response). Tumors were assessed by computed tomography or magnetic resonance imaging at baseline and every 8 weeks after randomization.

CELESTIAL trial showed that cabozantinib increased the median overall survival as compared to placebo from 8.0 months to 10.2 months (HR = 0.76, P 0.005) (31). The difference was more pronounced when the analysis was limited to patients whose only prior therapy was sorafenib (median overall survival: 11.3 vs. 7.2 months). The median progression-free survival was also higher on cabozantinib, 5.2 months as compared to 1.9 months on placebo (HR = 0.44, P <0.001). The most common grade 3 or 4 AEs with cabozantinib were palmar–plantar erythrodysesthesia (17% vs. 0% in the placebo group), hypertension (16% vs. 2%),

increased aspartate aminotransferase (12% vs. 7%), fatigue (10% vs. 4%), and diarrhea (10% vs. 2%) (31).

The success of the clinical trial for cabozantinib expands the agents available for HCC therapy as second-line treatment. Based on these findings, in January 2019, cabozantinib was approved for treatment of patients with HCC who have been previously treated with sorafenib. Consensus-based guidelines from the NCCN recommend considering cabozantinib only for patients with CP A cirrhosis.

ADVERSE EFFECTS AND MANAGEMENT

Within clinical trials, AEs were graded according to severity—Grade 0 (none), grade 1(mild), grade 2(moderate), and grade 3 (severe). Grade 0 to grade 2 AEs did not require any change to the treatment regime. Grade 3 AEs necessitated an interruption to treatment until improvement in symptoms. Patients with grade 3 AEs were also given reduced doses if the event re-occurred on recommencing the medication.

Across the four sentinel phase III trials done for sorafenib, regorafenib, lenvatinib, and cabozantinib, a significant proportion of patients reported drug-related AEs. The incidence of grade 3 AEs ranged from 45% to 75% (5, 6, 26, 31). This indicates that a majority of patients would have experienced an interruption to their treatment or dose reduction because of AEs. The most common AEs and their management recommendations are detailed below.

Hand–foot syndrome (HFS)

Hand–foot syndrome is also known as palmar–plantar erythrodysesthesia. It is a common reaction to TKIs which can occur within days of commencing the drug. In some cases, presentation is delayed and can commence several months after the initiation of the drug. It is most commonly seen with regorafenib, occurring in 53% of patients (26). It was also the most prevalent AE in sorafenib, lenvatinib, and cabozantinib trials, occurring in 21%, 27%, and 46% of patients, respectively (4, 5, 30). The hands and feet are frequently involved. Symptoms include altered sensation (numbness and tingling), stiffness, and pain. Erythema is often seen with some patients also experiencing hyperkeratosis or onycholysis. HFS can affect patients' ability to perform activities of daily living, impairing the quality of life (32).

Diagnosis is made clinically. Recommendations for the management of HFS are largely derived from clinical experience rather than trials. Prophylactic use of emollients and urease-based creams three times a day results in decreased incidence of HFS (33). Other strategies include avoiding mechanical trauma to hands and feet in the form of friction or extreme temperatures. This entails wearing well-fitting shoes with padded insoles and using non-foaming cleansers (33).

If HFS develops, topical corticosteroid cream and topical lignocaine are recommended for symptomatic relief. Oral analgesics can be used with caution. Cessation or dose reduction of the drug leads to improvement in symptoms but this is not ideal from a HCC perspective. Temporary cessation and re-introduction of the drug when symptoms have completely resolved is recommended. If severe symptoms recur, a dose reduction or discontinuation may be necessary.

Diarrhea

Diarrhea is the second most common AE. It is strongly associated with sorafenib and cabozantinib, occurring in 55% and 54% of patients, respectively (33). Initial management includes cessation of lactulose and making dietary changes to avoid food triggers. Sufficient fluid intake should be emphasized to ensure patients do not become dehydrated. When the above strategies are insufficient to manage symptoms, loperamide is recommended, with a maximum dose of 16 mg per day.

Hypertension

Hypertension is a common side effect of all TKIs. Patients taking lenvatinib and regorafenib had the highest incidence of hypertension (42% and 31%, respectively) and the largest number of patients with grade 3 hypertension (23% and 15%). In comparison, only 5% of sorafenib-treated patients reported hypertension. Of these, 2% of patients had grade 3 hypertension (33).

It is recommended that all patients have their blood pressure checked prior to commencing TKI treatment and be monitored throughout their treatment course. Angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), and beta blockers are all appropriate choices for the management of hypertension.

Fatigue

It is difficult to differentiate whether the fatigue reported by patients is due to TKIs or may be a symptom of advanced HCC and cirrhosis. Fatigue may also be associated with malnutrition caused by other TKI-associated AEs. Physical exercise has been shown to reduce fatigue in patients with advanced malignancy (33). Management is largely supportive in the form of encouraging adequate rest and nutrition.

Nausea and vomiting

Nausea, vomiting, decreased appetite, and weight loss are the common side effects of TKIs. The prevalence of nausea was highest in patients on cabozantinib (31%) and sorafenib (24%). Although relatively common, nausea and vomiting were rarely severe with less than 2% of patients across all studies experiencing grade 3 nausea and vomiting (33). Antiemetics can be utilized for symptom control. Ondansetron can cause QT prolongation and should be used with caution in combination with sorafenib, cabozantinib, and lenvatinib, as these agents can also cause QT prolongation.

CONCLUSION

TKIs have been the mainstay of systemic treatment for advanced HCC since 2007. Although AEs limit their usage, patients who can tolerate TKIs do have a survival benefit. The landscape in the management of advanced HCC has been changing

rapidly over the past few years. With the new arsenal of therapies available for advanced HCC, sorafenib is no longer the sole therapeutic option. Lenvatinib, the new first-line agent for unresectable HCC patients, though non-inferior to sorafenib in terms of overall survival, did demonstrate significantly improved progression-free survival and objective response rates, while also being generally well tolerated. Either option is reasonable for selection by the treating physician. Similarly, second-line options are also available now. While these agents provide a multitude of additional therapeutic options, some questions remain to be addressed. The data on second-line agents have been reported in patients who had prior sorafenib and not lenvatinib. The choice of second-line agents may be based on various factors including physicians' comfort, familiarity with using a particular agent, and patient choice after education regarding safety profile. Finally, the impact of various combination therapies on advanced HCC is currently being investigated. Combination therapies with TKIs and PD-1 inhibitors are currently in their early phase and are being evaluated in terms of safety and tolerability. These ongoing developments will certainly go a long way. Although great strides have been made, ongoing progress is still needed and yet to come.

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8

Multidrug Resistance in Hepatocellular Carcinoma

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Abstract: Although there has been tremendous progress in the treatment of hepatocellular carcinoma over the past decades, multidrug resistance to chemotherapy and targeted therapy remains a major hindrance in its successful management. Multidrug resistance, whether intrinsic or extrinsic, is a multifactorial process that includes enhanced drug efflux, decreased drug uptake, intracellular sequestration, metabolic alterations, aberrant apoptotic and autophagic signaling, changes in tumor microenvironment, and acquisition of stem cell-like properties by the cancer cells. Although many experimental strategies have been developed to overcome drug resistance, translation of the knowledge to the clinic has not been crowned with success. This chapter provides an overview of the role of multidrug resistance in hepatocellular carcinoma and the potential approaches to overcome this obstacle.

Keywords: ATP-binding cassette transporter; drug efflux; drug sequestration; multidrug resistance; RNAi therapy

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INTRODUCTION

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is increasing in prevalence with a high mortality rate. It is considered the fifth most detected cancer in men and seventh in women in the USA, and represents the third most leading cause of cancer-related death in the world. The highest incidence rate of liver cancer in the world occurs in Asia and Africa; hepatitis viruses (B and C) account for approximately 80% of all HCC cases (1). About 80% of HCC patients are currently diagnosed at advanced stages of the disease and are not suitable candidates for surgical resection of the tumor. Systemic chemotherapy with cytotoxic agents (5-Fluoracil, doxorubicin, cisplatin, and oxaliplatin) and targeted therapy with the tyrosine kinase inhibitor sorafenib are the main approaches for these patients; however, chemotherapy resistance remains a major clinical obstacle (2). In addition to drug resistance, sorafenib failed to be an optimal treatment modality for some advanced HCC patients due to adverse effects and high costs (1). Extensive studies have been carried out in the last few decades to enhance the efficacy of anticancer drugs by overcoming chemoresistance, but translating this knowledge to the clinic still represents a critical challenge. Thus, there is an urgent need to focus on elucidating the mechanisms of chemoresistance, especially multidrug resistance (MDR), and develop novel methods or tools for the treatment of HCC patients.

MECHANISMS OF MDR

MDR can be either intrinsic or acquired. In intrinsic resistance, the cancer cells are inherently resistant or unresponsive to therapeutics. In acquired resistance, cancer cells that were initially responsive become unresponsive during the course of treatment. MDR is multifactorial, and pleiotropic cellular signals are simultaneously involved in this process. These include upregulation of drug efflux, downregulation of drug uptake, sequestration of drugs, alteration in drugs metabolism, abnormal expression of non-coding RNAs, blockage of apoptotic signals, change of tumor environment, acquiring stem-cell like characteristics and autophagy (Figure 1) (3). More than one MDR mechanism can occur in a single cancer type, which pose significant challenges for a thorough understanding of the signaling network (4).

Enhanced drug efflux

Molecular pumps that transport cytotoxic drugs across the membrane of cancer cells represent a primary cause of chemotherapeutic resistance. Hyperactivation of these molecular pumps decreases intracellular drug concentrations and results in drug resistance. Permeability-glycoprotein, also referred to as P-gp, MDR-1, or ATP-binding cassette subfamily B member 1 (ABCB-1), is a well-studied 170 kDa plasma membrane drug efflux protein. It belongs to the adenosine triphosphate binding cassette (ABC) transporter superfamily, which includes MRP-1 (MDR protein), TAP1 (Transporter 1, ATP-binding cassette subfamily B member), and

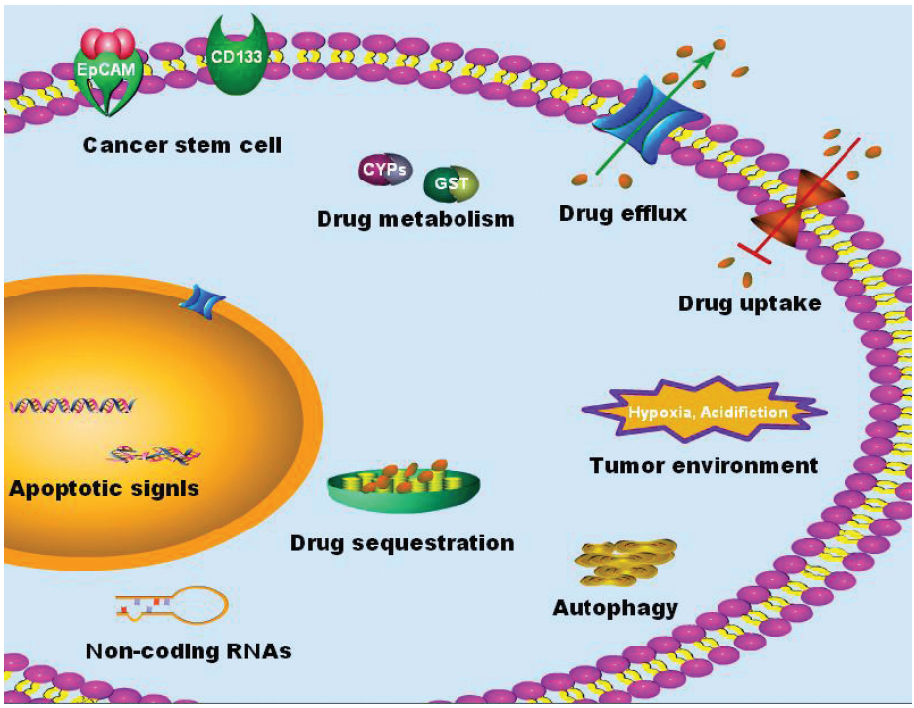

Figure 1 Multiple mechanisms of MDR in HCC. Multidrug resistance is a multifactorial process. Some of these include enhanced drug efflux, reduced drug intake, alterations in tumor microenvironment, impaired autophagy and apoptotic signals, lysosomal sequestrations, non-coding RNAs, alterations in drug metabolism, and acquisition of cancer stem cell-like phenotypes. CYPs, cytochrome P450 enzymes; GST, glutathione-S-transferase.

BCRP (breast cancer resistance protein) (5). ABC transporters are members of a conserved family of transmembrane proteins that utilize ATP as energy source to transport various substances, such as metabolic products, sterols, lipids, and drugs, across cellular membranes. The ABC proteins are comprised of cytosolic and transmembrane domains (6), and are essential for normal cellular functions. However, overexpression of ABC proteins in cancer cells usually leads to insufficient intracellular concentration and bioavailability of cytotoxic drugs as well as their metabolites (7). From a pharmacological point of view, although drug molecule–ABC interactions are very specific, one drug moiety can be a substrate of several ABC pumps (8). ABC proteins play a major role in the MDR of HCC (9, 10). The drug-resistant HCC cell line Bel7402/5-FU, developed by exposure to increasing concentrations of 5-FU, displays a higher expression of P-gp when compared with the parental cell line Bel7402. These cells are resistant not only to 5-FU but also to epirubicin (11). Kong et al. showed that P-gp and BCRP were highly expressed in the MDR HCC cells HepG2, which was induced by TGF-β1 via the SMAD4/HOTAIR/MiR-145 axis. As a result, the concentration of imatinib in HepG2 cells was significantly decreased (12). Compared with parental cells, P-gp is significantly overexpressed in the sorafenib-resistant HCC cells, HepG2

and Huh7. This was partially due to epithelial-mesenchymal transition (EMT) and AKT activation. Treatment with the novel allosteric AKT inhibitor MK-2206 reversed P-gp-mediated MDR via downregulation of phosphorylated AKT (13).

Reduced drug uptake

Drugs are transported across the cells by several mechanisms including passive diffusion and facilitated transport. The plasma membrane is an important barrier that limits drugs from reaching intracellular compartments. Passive transporters, ion-coupled transporters, and exchangers are encoded by genes of the solute carrier (SLC) family, which comprises approximately 360 uptake transporters in the cell membrane. Factors that downregulate or block the transporters can lead to drug resistance through decreased drug uptake or defective endocytic processes (14). Compared with non-tumor adjacent tissues, SLC46A3 was downregulated in 83.2% of human HCC tissues, and low expression was associated with a more aggressive phenotype. Conversely, overexpression of SLC46A3 was demonstrated to ameliorate sorafenib resistance, thereby improving the drug response, both in vitro and in vivo (15). SLCO1B3 is involved in the uptake of a number of chemotherapeutic agents, and its expression is significantly elevated in HCC patients with intratumoral cholestasis (16, 17). As a direct target of miRNA122, SLC7A1 is upregulated in miR122-silenced HCC cells, which is related to sorafenib resistance. Overexpression of miR122 can suppress SLC7A1 levels and render HCC cells more sensitive to sorafenib (18). Gao et al. analyzed SLC family genes using qPCR array and identified 11 downregulated and 3 upregulated genes in HCC specimens, compared with the para-carcinoma tissues from HCC patients who underwent surgery. In addition, they found that SLC29A1 was the only gene that correlated with poor prognosis and that it was significantly elevated in human HCC cell lines and tissues. Knockdown of SLC29A1 decreased the sensitivity of HCC cells to 5-FU, cisplatin, and sorafenib in vitro (19).

Drug sequestration

Sequestration of drugs in cellular compartments is an important mechanism of chemotherapy resistance. Since drugs used in chemotherapy generally target molecules in the nucleus and other subcellular compartments, they must be able to achieve sufficient concentrations in these compartments and their microenvironments (20). Intracellular conditions such as intralumenal pH, electrochemical potential, lipid compositions, and resident proteins can influence the intracellular localization of drugs. Multiple drug sequestration mechanisms may be involved in a single MDR cancer cell line, and the phenomena of drug sequestration may be more complex than originally thought (21). MDR cell lines show an increased capacity to sequester drugs into cytoplasmic compartments, resulting in decreased interactions of the drug with its nuclear targets. Colombo et al. (22) demonstrated P-gp expression not only on the cell membrane but also on lysosomes of six HCC cell lines and reported that cell lines with giant lysosomes were more resistant to sorafenib than those with small lysosomes. They concluded that lysosome-associated drug sequestration plays an important role in MDR in HCC cells (22). Metallothionein also plays a role in sequestering drugs

within a cell. Sorafenib remarkably induces the expression of metallothionein-1G, which is a critical factor for sorafenib resistance in HCC. Inhibition of metallothionein-1G enhances the anticancer activity of sorafenib in vitro and in tumor xenograft models (23).

Cellular metabolism

The response to cytotoxic drugs often depends on the metabolic state of the cancer cells, and these cells rewire the metabolism of anticancer drugs. Metabolic alterations can be influenced by various factors such as oncogenes or tumor suppressor genes and the tumor microenvironment (24–29). Cancer cells that are resistant to cisplatin have high levels of reactive oxygen species (30), glutathione (GSH), and glutamate–cysteine ligase catalytic subunit (GCLC) (31, 32). Downstream of survival signaling pathways, the Warburg effect, which refers to the increased rate of glycolysis in tumorigenic cells, can be observed even in conditions of normal oxygen levels. In c-Myc-driven HCC, glucose catabolism through glycolysis is elevated via the activation of pyruvate kinase (33). Inhibition of glycolysis and increase in oxidative phosphorylation can re-sensitize HCC cells to chemotherapeutics such as sorafenib, cisplatin, and isoliensinine (34). HIF1α activates the transcription of genes encoding angiogenic cytokines, for example, VEGF, and glycolytic enzymes, such as hexokinase 1, hexokinase 2, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase. These enzymes rewire the metabolism of cancer cells and induce MDR in HCC (35–38). Wu et al. showed that ADRB2 pathway regulation leads to HIF1α stabilization, reprogramming of glucose metabolism, and resistance of HCC cells to sorafenib (39). Drug metabolism enzymes are also involved in the MDR. This process includes phase I and phase II enzymes. Phase I of oxidative metabolism is mediated mainly by cytochrome P450 enzymes (CYPs) and epoxide hydrolases. Phase II enzymes are involved in conjugation reactions, including glutathionylation, glucuronidation, and sulfation. These enzymes include glutathione-S-transferase (GST), UDP-glucuronosyltransferases (UGT), sulfotransferases, and arylamine N-acetyltansferases (NAT), which transform the reactive species into hydrophilic nontoxic metabolite conjugates. Therapeutic drugs are metabolized by CYPs and epoxide hydrolases, which are further conjugated by the phase II enzymes and then, in phase III, effluxed by transporters such as the members of the ABC transporter family (14). Meena et al. reported that CYP450 and fatty acid synthase protein levels were elevated in multidrug-resistant HCC cells, and downregulation of these molecules by siRNAs or cerulenin resensitized the cells to paclitaxel (40). Further, ciplatin-resistant HCC cell lines have a higher expression of GST, which can protect cancer cells from being inhibited by anticancer drugs (41).

Non-coding RNAs

The term “non-coding RNAs” (ncRNAs) refers to RNAs that do not encode proteins. These include miRNAs, long ncRNAs (lncRNAs), and circular RNAs (circRNA) (42). ncRNAs are involved in multiple cellular processes, such as proliferation, migration, apoptosis, angiogenesis, and immune responses (43).

A number of studies have highlighted the key roles of ncRNAs in the evolution and progression of drug resistance in cancers. They mainly modulate drug transporters, cell cycle-related proteins, apoptotic signals, and the tumor microenvironment (44). While all ncRNAs potentially play roles in drug resistance in a context-specific manner, the major role is played by miRNAs and lncRNAs (45, 46). The miRNAs are small (~20 bp) non-coding RNAs, which target specific mRNA sequences and inhibit protein translation (47).

One of the most abundantly expressed miRNA in the liver is miR-122, which plays a major role in basic liver function and homeostasis (48, 49). The loss of miR-122 is attributed to dysregulation of hepatocyte differentiation, poor prognosis, and metastasis of liver cancer. Restoration of miR-122 increased the sensitivity of drug-resistant HCC cells to cytotoxic agents through downregulation of MDR-related genes, and inhibition of cell growth by cell cycle arrest at G0/G1 phase (50). Moreover, miRNA microarray data indicate that miR-122 is decreased in sorafenib-resistant HCC cells. miR-122 downregulation-mediated activation of insulin-like growth factor 1 and subsequent activation of the RAS/RAF/ERK pathway are thought to be the major mechanisms of resistance (51). He and colleagues found that miR-21 was overexpressed in sorafenib-resistant HCC cells, and inhibition of miR-21 with oligonucleotides resensitized these cells to sorafenib (52). They concluded that miR-21 participated in the acquired resistance of sorafenib by suppressing autophagy through the Akt/PTEN pathway (52). Multidrug-resistant Huh-7 cell lines, developed with increasing concentrations of doxorubicin, cisplatin, carboplatin, mitomycin C, and vincristine, demonstrated a significant differential profile of miRNAs when compared with the parental cell line. miR-27b, miR-181a, miR-146b-5p, miR-181d, and miR-146a were the most differentially expressed, and they are thought to play critical roles in the acquisition of MDR by regulating PTEN, P53, and KRAS (53).

Apoptotic signals

Apoptosis is involved in the regulation of many physiological and pathological processes (54). Disruption of apoptotic signals, one of the hallmarks of cancer, is a major obstacle in the success of chemotherapy. In general, there are two apoptotic pathways: (i) the intrinsic pathway involving the release of cytochrome c from mitochondria and (ii) the extrinsic pathway with the activation of death receptors. The initiation of these pathways results in the activation of caspases, which mediate the cleavage of cellular substrates, leading to morphological and biochemical changes that accompany apoptosis (55). DNA damage and oncogene activation either induce the accumulation of p53, which causes cell cycle arrest in the G1 phase, or trigger apoptosis, depending on the extent of DNA damage. Mutation or inactivation of p53 can result in chemotherapy resistance in cancer via suppression of apoptotic pathways (10). Zhang et al. reported that cisplatin reversed tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) resistance in HCC cells, dependent on the status of p53 (56). Modulating the expression of p53 and BCL-2 using long interspersed nuclear element-1 ORF-1 protein led to the resistance of HepG2 cells to cisplatin and epirubicin in vitro (57). The BCL-2 family, including pro-apoptotic proteins (BAX, BAK, BID, BAD, and PUMA) and anti-apoptotic proteins (BCL-2, BCL-xl, and MCL-1), can

regulate apoptosis induced by wild-type p53 in response to stress. Mitochondrial pathway-associated chemotherapy resistance is mainly regulated by the BCL-2 family (14). BCL-2 plays a pivotal role in the glycochenodeoxycholate (GCDA)-induced chemoresistance, while suppressing the GCDA-stimulated phosphorylation of BCL-2 significantly attenuates the survival and drug resistance in HCC cells (58). Sorafenib-resistant HCC cell lines, including HepG2R and Hep3BR, exhibit altered expression of BCL-2 and MCL-1. Navitoclax, an inhibitor of BCL-2, can restore the anticancer activity of sorafenib and regorafenib via a mitochondrial caspase-dependent mechanism in vitro and in vivo (59).

Tumor microenvironment

Solid tumors are heterogeneous structures. The tumor microenvironment is composed of cancer and stromal cells embedded in extracellular matrix, sustained by aberrant vasculature (60, 61). Tumor hypoperfusion, secondary to the hyperpermeability of the aberrant vasculature, along with low oxygen, depleted nutrition, low pH, and high interstitial pressure can cause chemoresistance (61, 62). Compared to normal cells, cancer cells exhibit higher glucose metabolism rates and preferentially utilize glycolysis over oxidative phosphorylation, especially in hypoxic conditions (Warburg effect). This process ultimately generates lactic acid, leading to intracellular acidification (63, 64). As a result, cancer cells may express relatively more proton pumps in order to maintain intracellular pH homeostasis, rendering the extracellular environment highly acidic. According to the ion trapping theory, weakly basic drugs, such as doxorubicin, mitoxantrone, and vincristine, are ionized extracellularly and, as a consequence, lead to chemoresistance (14). Being an anti-angiogenic agent, sorafenib treatment reduces tumor vessels, prompts hypoxia in the tumor microenvironment, and stimulates HIF-mediated cellular responses that favor the selection of chemo-resistant cells (65). Hypoxia has been shown to induce resistance to sorafenib, 5-FU, gemcitabine, cisplatin, adriamycin, and 6-thioguanine in BEL-7402, HepG2, and SMMC-7721 HCC cell lines (66).

Cancer stem cells

Cancer stem cells (CSCs) are a subpopulation of tumor cells with the capacity of self-renewal, differentiation, as well as drug resistance (14, 67, 68). CSCs in human HCC have been identified and validated through isolation and xeno-transplantation experiments in animal models. These cells have pivotal roles in the development and progression of HCC (69) as well as chemotherapy resistance (66). CSC markers of HCC include epithelial cell adhesion molecule (EpCAM), CD133, CD90, CD44, CD24, CD13, deubiquitinating enzyme ubiquitin-specific protease 22 (USP22), and oval cell marker OV6. Some of these markers have been reported to confer chemoresistance to HCC (2, 70, 71). Multi-signal pathways and their cross-talk, including EpCAM, Wnt/β-catenin, Sonic Hedgehog, and Notch, are required to maintain the stemness phenotype of HCC CSCs (67). $CD133^+$ HCC cells isolated from human HCC cell lines and xenograft mouse models were resistant to chemotherapeutics, through the activation of Akt/PKB and Bcl-2 pathways (72). Downregulation of USP22 significantly suppressed the expression of ABCC1 (MRP1) in an HCC cell line, with validation of the

relationship between USP22 and ABCC1 in clinical HCC tissue samples. These results suggest that USP22 is associated with the MDR phenotype of BEL-7402/FU (71). In addition, GSK2879552 and pargyline, inhibitors of lysine-specific histone demethylase 1A (KDM1A or LSD1), were demonstrated to alleviate acquired resistance to sorafenib through the suppression of the Wnt/β-catenin signaling pathway in HCC CSCs (73).

Autophagy

Autophagy is a highly conserved cellular "self-degradative" process, in which cytoplasmic components (long-lived or misfolded proteins, protein aggregates, and damaged organelles) are degraded and recycled to maintain homeostasis. Deficient autophagy is closely related to the development of many diseases including cancer. Autophagy occurs at a basal level in cells and can be induced by diverse signals and cellular stressors, including chemotherapeutic agents (74). In general, autophagy plays a dual role in the process of MDR in cancers. It not only contributes to the development of MDR, but also kills MDR cancer cells in which apoptosis pathways are inactive, leading to inconsistence results across studies (75, 76). Autophagy inhibitors can increase the sensitivity of HCC cells to cytotoxic agents (77). Fan et al. showed that elevated peptidylarginine deiminase IV (PADI4) was associated with chemoresistance through autophagy induction in HCC in vitro and in vivo. Inhibition of autophagy restored the sensitivity of HCC cells to chemotherapy (78). The exact relationship between autophagy and MDR in HCC remains unclear and requires further research.

STRATEGIES TO OVERCOME MDR

Extensive studies have been carried out during the last few decades to enhance the efficacy of chemotherapy by suppressing or evading the mechanisms of MDR. These approaches include the use of MDR modulators or chemosensitizers (79, 80), improved drug delivery (81, 82), RNAi therapy (83), and natural products (84).

MDR modulators or chemosensitizers

MDR modulators or chemosensitizers have been classified into first-generation, second-generation, and third-generation on the basis of their affinity for certain transporters and effects (5). As P-gp is the most extensively characterized transporter of the ABC superfamily, ways to modulate P-gp have been studied extensively. The first-generation P-gp modulators include verapamil, cyclosporine A, trifluoperazine, quinidine, progesterone, calmodulin antagonists, and tamoxifen. Kim et al. reported that a high dose of verapamil is required both clinically and experimentally to overcome MDR of HCC and that the combination of tamoxifen and cyclosporine A showed a significant reduction in the IC_{50} value of doxorubicin in MDR HCC cell lines (85). Due to disappointing therapeutic outcomes and high systemic toxicities, these modulators were replaced with the second-generation MDR modulators (86, 87) such as dexverapamil, valspodar,

biricodar citrate, and dexniguldipine. Valspodar was shown to improve the anticancer effect of doxorubicin by modulating P-gp in HCC and hepatoblastoma cell lines (88). Although the second generation of MDR modulators can inhibit P-gp and increase the intracellular accumulation of drugs better than the first-generation modulators, there are several disadvantages that limit their clinical application. Numerous chemotherapeutics are substrates of both P-gp and cytochrome P450. Thus, the combination of anticancer agents with the second-generation MDR modulators may lead to unpredictable pharmacokinetic or incorrect dosing of chemotherapeutics (5, 89). The third-generation MDR modulators include tariquidar, zosuquidar, laniquidar, elacridar, mitotane, diarylimidazole, and annamycin. Comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) associated with 3D-quantitiative structure–activity relationship (3D-QSAR) studies were performed to aid the research and design of the third-generation MDR modulators (90). These modulators are about 300 times more potent than the first- and second-generation modulators. Importantly, these agents do not interact with cytochrome P450 (90, 91). Takahata et al. found that breast cancer-resistant protein (BCRP) expression correlated well with the chemo-sensitivity of irinotecan hydrochloride (CPT-11) in HCC cell lines. Elacridar, an inhibitor of BCRP, enhanced the sensitivity of CPT-11 in BCRP-overexpressing KYN-2 cells (92).

Enhanced drug delivery

Nanotechnology has the power to deliver anticancer drugs and radically change chemoresistance of cancer cells by overcoming MDR (82). There are several drug delivery systems including liposomes, dendrimers, polymeric micelles, nanoparticles, polymer–drug/protein–conjugates, and carbon nanotubes. These nanoformulations may overcome several challenges in efficient drug delivery such as solubility, pharmacokinetic profiles, cellular uptake, bio-distribution patterns, circulation times, and clearance (93). For instance, pluronic P85 can sensitize MDR tumors to many chemotherapeutic agents through various mechanisms: (i) membrane fluidization, (ii) ATP depletion, (iii) direct interaction with the ABC efflux transporter, (iv) reduction of the GSH/GST detoxification system, (v) drug release from acidic vesicles, and (vi) incorporation into the mitochondrial membrane, thereby inhibiting cellular respiration (94). Moreover, all these nanomaterial-based drug delivery systems can be conjugated with various kinds of ligands (e.g., proteins, antibodies, and small molecules) producing the so-called actively-targeted material that favors drug targeting to specific cell surfaces and thus to specific cell populations, leading to a selective and reduced toxicity (82).

Polyethylene glycol (PEG) and polyethylenimine (PEI) co-conjugated ultrasmall nano-graphene oxide (NGO) loaded with C6-ceramide (NGO-PEG-PEI/Cer) were reported to subvert MDR in HCC cells by inactivating MDR and AKT signaling. NGO-PEG-PEI/Cer combined with sorafenib represents a promising potential therapeutic strategy for the treatment of drug-resistant HCC (95). HA/anti-miR-21/PPAuNCs, a nonviral gene delivery system, which condensed anti-miR-21 into hyaluronic acid-conjugated and PEI-modified PEGylated gold nanocages (AuNCs), enhanced intracellular drug accumulation and restored sensitivity to doxorubicin in a doxorubicin-resistant HCC cell line through upregulating PTEN expression and downregulating P-gp (96). Bmi1 is essential for the survival

and proliferation of liver CSCs. Yang et al. demonstrated that Bmi1 siRNA delivered via cationic nanocapsules of cisplatin (NPC/Bmi1siR) eliminated the side population of $CD133^+$ HCC cells dramatically and overcame drug resistance of HCC (97).

RNAi therapy

RNA interference (RNAi) is considered a highly specific approach for gene silencing and has emerged as a novel therapeutic tool for various pathologic conditions, including cancers (98,99). RNAi molecules are a group of small regulatory RNAs that include miRNAs and small (or short) interfering RNAs (siRNAs). miRNAs are endogenous RNAs that are produced from non-coding RNAs, while siRNAs are derived from exogenous long dsRNAs (100, 101). In addition, exogenous short hairpin RNA precursors that are processed by a distinct cellular machinery to form siRNAs can also lead to effective gene silencing (101, 102). These artificially generated oligonucleotides mediate gene silencing through post-transcriptional mRNA cleavage and decomposition in the cytoplasm, resulting in the knockdown of target gene expressions (98, 103). Theoretically, RNAi-based strategies can be used in a wide variety of experimental models to target genes that are involved in disease processes (103, 104).

Enhancer of zeste homolog 2 (EZH2) is overexpressed in the MDR HCC cell line Bel/Fu, and siRNA depletion of EZH2 sensitized these cells to 5-FU by inhibiting MDR1 protein expression, promoting apoptosis, and inducing cell cycle arrest at G1/S phase (105). It has been reported that MAPK14/Atf2 signaling predicted a poor response to sorafenib in human HCC. Rudalska et al. demonstrated that silencing MAPK14 by shRNA reverted sorafenib resistance in HCC in vitro (106). Knockdown of the autophagy-related gene LC3 by RNAi significantly enhanced the sensitivity to epirubicin and inhibited proliferation of HepG2 cells (107). As silencing a single miRNA may sequentially activate other compensatory signaling pathways, a combinatorial approach modulating many miRNAs related to a signal pathway may be a promising strategy. The miRNAs miR-21, miR-153, miR-216a, miR-217, miR-494, and miR-10a-5p have been shown to be elevated in sorafenib-resistant HCC cells. Simultaneous targeting of these miRNAS using artificial long non-coding RNAs reversed sorafenib resistance in these cells both in vitro and in vivo (108). RNAi, apart from being a potential therapeutic tool, can also be used as a tool for biomarker screening for chemotherapy sensitivity. Through a high-throughput RNAi screening with 176 shRNA pools against 88 histone methyltransferases and histone demethyltransferases, Li et al. (109) found that silencing of the histone methyltransferases genes, *ASH1L, C17ORF49,* and *SETD4,* promoted the sensitivity of HepG2 cells to sorafenib.

Natural products

Natural products have attracted increasing attention as anticancer tools. A large pool of products with potential functions on reversing MDR have been identified and classified (Figure 2) (84, 110). Steroidal saponin from *Trillium tschonoskii* reversed MDR of HCC cell lines in a dose-dependent manner by inhibiting MDR-related molecules such as MRP1, MRP2, MRP3, MRP5, MVP, and GST-π (111).

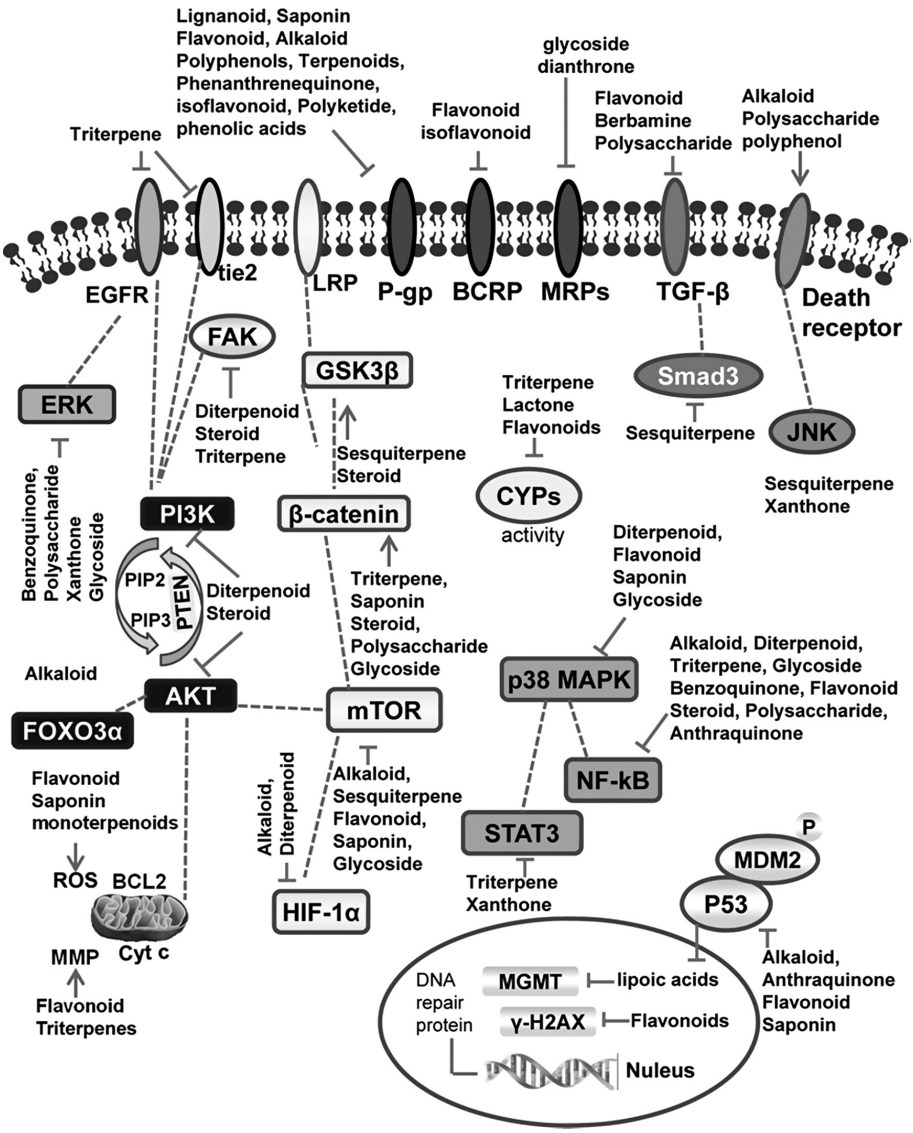

Figure 2 Natural products and their potential role in reversing MDR. Experimental data show that natural products can reverse MDR via regulating drug efflux, drug metabolism, and apoptotic pathways in cancer cells (110).

Treatment of HepG2/ADR cells with rhamnetin, derived from Persian berries, reduced the expression of Notch-1, P-gp, and BCRP and increased the susceptibility of HepG2/ADR cells to sorafenib, etoposide, and paclitaxel (112). Baicalein, isolated from *Radix scutellariae,* increased the intracellular accumulation of Rho123 and epirubicin, induced apoptosis and autophagy, decreased the expression of P-gp and Bcl-xl, and reversed MDR in Bel7402/5-FU cells (11).

Moreover, natural products can also increase the sensitivity of HCC cells to anticancer drugs by regulating cellular metabolism. Li and colleagues demonstrated that dauricine dose-dependently suppressed glucose glycolysis and increased oxidative phosphorylation by downregulating the expression of hexokinase 2 and pyruvate kinase M2, consequently increasing the sensitivities of HCC to cisplatin, sorafenib, and isoliensinie (34).

CONCLUSION

Despite a better understanding of the mechanisms of drug resistance, and the experimental approaches that have been taken to overcome drug resistance over the decades, clinical utility of these approaches has not come to fruition. To date, there is no effective tool to overcome MDR of HCC patients. Among the various strategies described to address drug resistance, nanotechnologies appear to offer particular advantages with their presumed target-specific delivery of chemotherapeutics and other conjugated agents. While RNAi can be designed for specific targets and used successfully in vitro, the in vivo silencing effects of RNAi are far from satisfactory even in highly controlled experimental conditions. Natural products can affect multiple targets and pathways with minimal side effects. However, the current literature is not sufficient to justify their use in clinical settings. Given that the liver plays a major role in drug metabolism and detoxification, and its function is already impaired in HCC patients, any drug combination that depends on normal liver metabolism is unlikely to be a successful strategy to overcome drug resistance. Taken together, continuous efforts are needed to explore the mechanisms in more detail and design novel approaches to overcome MDR to improve outcomes for HCC patients.

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